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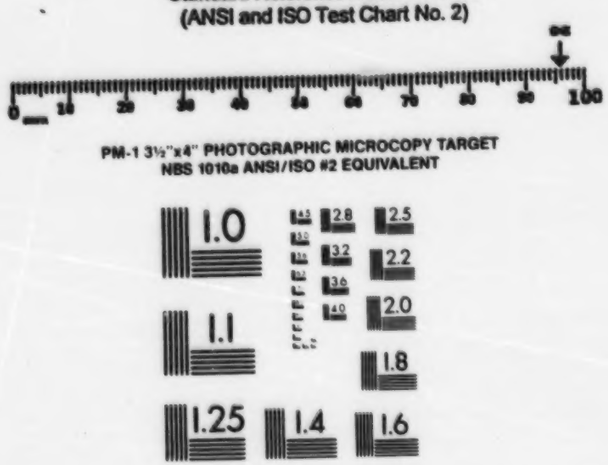

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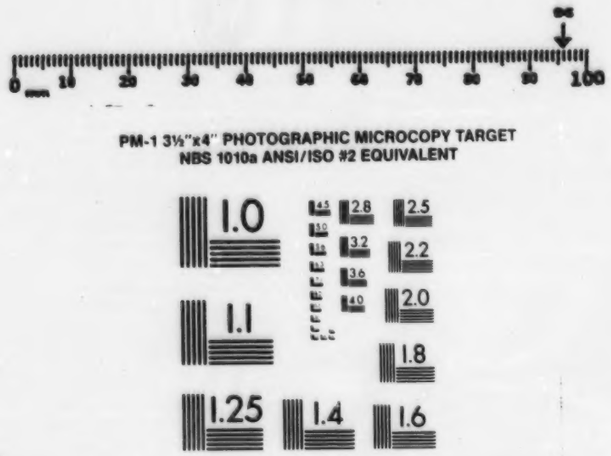

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FINAL REPORT

19980030

BIOTECHNOLOGY FOR SASK PULSE IMPROVEMENT PROGRAM - CDC

August 2002
Funded by the Agri-Food Innovation Fund

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Canada

Saskatchewan

FINAL REPORT
AFIF PROJECT #98000030
March 31, 2002

"BIOTECHNOLOGY FOR SASKATCHEWAN PULSE CROP IMPROVEMENT"

Compiled by
Dr. Bert Vandenberg
Crop Development Centre
University of Saskatchewan
51 Campus Drive
Saskatoon SK S7N 058

On behalf of:
Dr. A. McHughen (CDC)
Dr. A.E. Slinkard (CDC)
Dr. T. Warkentin (CDC)
Dr. L. Buchwaldt (AAFC)

In association with:

Dr. Monika Lulsdorf (CDC)
Dr. Abebe Tullu (CDC)
Dr. Bunyamin Ta'ran (CDC)

And in collaboration with

Dr. P. Fobert (PBI)
Dr. J. Mahon (PBI)

AFIF PROJECT #98000030
"BIOTECHNOLOGY FOR SASKATCHEWAN PULSE CROP IMPROVEMENT"

FINAL PROJECT REPORT – MARCH 31, 2002

PROJECT SUMMARY – see individual sub-projects

TECHNICAL REPORT

BACKGROUND AND OBJECTIVES

This research project was originally designed as a collaborative research effort by the Plant Biotechnology Institute (PBI) of the National Research Council of Canada, the Crop Development Centre (CDC) of the University of Saskatchewan) and AgrEvo Canada Inc. It originally comprised 5 Sub-Projects, supervised by scientific staff from PBI and/or CDC. Sub-Projects 1 and 2 involve development of molecular markers for lentil and chickpea disease resistance genes and development of a lentil transformation system, respectively. The complete report on the progress of Sub-Project 5 will be provided by Dr. John Mahon of PBI as part of Project # 96000413. Sub-Project 3 was discontinued at the end of January 1999. It was replaced by Sub-Projects 6 and 7, which involve research into development of molecular markers for anthracnose resistance in lentil, and development of doubled haploid technology in chickpea, respectively. In early 2001, approval was granted to extend the project until March 31, 2002.

This report summarizes research results and research progress to March 31, 2002. Some significant personnel changes occurred during the course of the project. Dr. Tom Warkentin joined the CDC on July 1, 1999, as a pulse crop research scientist. His involvement in the project overlaps that of Dr. Al Slinkard, particularly in the area of chickpea and pea research. In 1999, Dr. Lone Buchwaldt became involved in the management of research involving molecular markers for disease resistance in lentil (Sub-Projects 1 and 6). Dr. Abebe Tullu joined the project on December 1, 1999 to lead the research on anthracnose markers (Sub-Project 6). Dr. Mahboob Chowdhury left the project in January 2000 for a position in Ontario. Dr. Bunyamin Ta'ran joined the project in February, 2000 to lead the research on ascochyta markers (Sub-Project 1). Dr. Ta'ran took a leave of absence from the project from May 1 – October 31, 2000 for personal reasons but resumed his position in 2001.

This project report is presented on a Sub-Project basis. Research outputs in the form of published papers, conference proceedings and posters are listed separately for each Sub-Project. Copies of all posters will be forwarded in CD format by the end of April 2002.

SUMMARY OF PROJECT GOALS AND OBJECTIVES

The original collaborative research project had the objective of developing new biotechnology capabilities and applying them to the genetic improvement of Saskatchewan pulse crops. The specific goals included:

- identifying and using molecular gene markers to combine disease resistance genes for ascochyta in lentil and chickpea, and for anthracnose in lentil,
- developing a method to produce transgenic lentils,
- producing of and testing of herbicide tolerance and anti-fungal genes in elite CDC pea breeding lines,
- finding new genes to turn on systemic disease resistance in peas,
- testing the stability and function of foreign genes and their effects on crop performance in transgenic peas and,
- developing a microspore system for production of doubled haploids in chickpea.

1 - TECHNICAL REPORT for SUB-PROJECT 1

Molecular Markers for Ascochyta Resistance in Chickpea and Lentil

Dr. A Vandenberg, Dr. T. Warkentin, Dr. L. Buchwaldt, Dr. B. Tar'an and Dr. A.E. Slinkard

1.1 Summary – Sub-Project 1

The research was conducted to identify molecular markers linked to the resistance genes for ascochyta blights in two populations of chickpea (P1279-2-1 x ICCV6-3 and CDC Chico x CDC Marengo) and lentil (Indianhead x Eston and ILL5588 x L692-16-1). A RAPD marker ($OPH04_{680}$) linked in repulsion with the resistance gene in the P1279-2-1 x ICCV6-3 cross was identified. A marker locus (SCAR $AFAG_{140}$) linked to the major gene and two additional loci with smaller effect to the reaction to ascochyta were identified in the second cross (CDC Chico x CDC Marengo) of chickpea. Flanking RAPD markers ($UBC227_{1290}$ and $OPD10_{870}$) were identified for the recessive gene controlling the resistance to ascochyta in Indianhead x Eston cross. Two loci tagged by $OPB18_{680}$ and $OPV1_{800}$ were associated with the resistance in the second population (ILL5588 x L692-16-1) of lentil. The markers with the largest effect to the resistance in each crop were converted into SCAR markers to simplify their analysis. The test on a collection of elite breeding lines demonstrated the potential use of these markers for assisted selection.

1.2 Background and Objectives – Sub-Project 1

Lentil and chickpea production has increased dramatically in Saskatchewan in the past few years. The rapid expansion of the crop acreage and shortened rotations increase the potential disease pressure from foliar pathogens such as ascochyta blights caused by *Ascochyta rabiei* and *Ascochyta lentis*. These diseases are major biotic stresses of chickpea and lentil production and pose a real threat to the sustainability of the crop production. The research was conducted to identify molecular markers linked to the resistance genes for ascochyta blights in chickpea and lentil. Field assessments and molecular marker analysis indicated that at least two different genes are present for resistance to ascochyta in chickpea and lentil. For example, P1279-2-1 and CDC Chico of chickpea that showed distinct resistance reaction to *Ascochyta rabiei* are believed to carry different genes. Furthermore, at least two resistance genes for *Ascochyta lentis*, one recessive derived from Indianhead and one dominant from ILL5588 are also present in lentil. The research was also designed to enhance our understanding of the genetic basis for resistance to ascochyta in chickpea and lentil. The results of the study will provide plant breeders with new tools to select for disease resistant plants and to pyramid disease resistance genes from different sources into elite breeding lines.

1.3 Results and Conclusions – Sub-Project1

Chickpea

1. Cross P1279-2-1 x ICCV6-3

P1279-2-1 is a desi type with moderate resistance to *Ascochyta rabiei* and ICVV6-3 is a highly susceptible small-seeded kabuli type. Following is the summary of the technical aspects and accomplishments for this cross. The genetic analysis was done in a F_2 generation from the cross between P1279-2-1 (desi type and moderately resistant to ascochyta) and ICCV6-3 (kabuli type and highly susceptible to ascochyta). The F_2 plants were screened for reaction to ascochyta under greenhouse conditions and scored on a scale of 1 (no symptom) to 9 (plants are dead). X^2 analysis demonstrated that the segregation ratio (13 resistant : 25 segregating : 18 susceptible) was not significantly different from the expected ratio of 1:2:1 ($P > 0.10$). Two DNA bulks, each constituted from 10 resistant and 10 susceptible lines, respectively, were screened for polymorphisms using more than 200 RAPD primers. Three primers produced reproducible polymorphic markers; however, only one primer generated a band ($OPH04_{680}$) that was closely linked (2 cM) in repulsion phase to the resistance gene derived from P1279-2-1. The $OPH04_{680}$ marker was then converted into a SCAR marker to simplify the analysis; however, the polymorphisms between the parents and the F_2 plants were lost when they were analyzed using this SCAR marker. Therefore, we suggested using the original RAPD marker ($OPH04_{680}$) for further application.

2. Cross CDC Chico x CDC Marengo

A segregating population was developed from a cross between CDC Chico (small kabuli; Moderately resistant to ascochyta) and CDC Marengo (large desi; highly susceptible to ascochyta). The reaction to ascochyta (on a scale of 0 to 9; where 0 = no symptom and 9 = plants are dead) was evaluated in both 120 lines of $F_{2:3}$ generation and 74 recombinant inbred lines ($F_{6:7}$ generation) under greenhouse conditions. Based on phenotypic evaluation, the $F_{2:3}$ lines were classified into 29 resistant, 67 segregating and 24 susceptible, while the RIL's were grouped into 32 resistant, 7 segregating and 35 susceptible. X^2 analyses indicated that these segregation ratios are not different ($P > 0.10$) from 1:2:1 and 1:1 expected ratios for the F_2 and $F_{6:7}$ generations, respectively. These results indicated that a major gene for resistance to ascochyta blight may be segregating in the population.

DNA was extracted from 110 F_2 plants and 74 RIL's. DNA samples from ten of each of the most resistant and susceptible lines were bulked. DNA's of the two parents and the resistant (R) and susceptible (S) bulks were screened for polymorphisms using 800 RAPD (Randomly Amplified Polymorphic DNA) primers, 50 ISSR (Inter-Simple Sequence Repeats) primers, 66 STMS's (Sequence-Tagged Microsatellite Sites) from chickpea and cowpea, and 48 AFLP (Amplified Fragment Length Polymorphisms) primer combinations. The STMS markers (co-dominant markers) for chickpea were developed by Dr. Peter Winter at the University Frankfurt and were used in the current study with permission.

No polymorphism between the R and S bulks was found from screening of all the RAPD and ISSR primers. Three STMS's (TA76s, TA110 and TA176) and one AFLP primer combination (EcoR+ACA and Mse1+CAC) produced polymorphic bands between the parents and the two bulks. These markers were then tested across each lines of the $F_{2:3}$ and $F_{6:7}$ generations. The association of these markers with the reaction to ascochyta blight was determined by one-way ANOVA and the results are presented in Table 1. The $ACA\text{-}CAC_{170}$ is

linked to the major locus for the resistance and captured 32 % and 46 % of the variation in the F_2 and RI populations, respectively. TA110 marker that was linked with the AFLP marker (ACA-CAC_{170}) at a distance of 16 cM on LG7 may be linked to the same gene for the resistance but at a farther distance than the ACA-CAC_{170} marker. Additional two loci on LG1 and LG4 that were tagged by TA76s and TA176 markers also contributed a smaller effect to the resistance to ascochyta in this population.

Table 1.1 Genomic location, percentage of the phenotypic variation and donor parent for marker loci associated with reaction to ascochyta blight in $F_{2:3}$ and RI populations of chickpea derived from a cross between CDC Chico and CDC Marengo

Marker locus	Linkage Group*	% variation ($F_{2:3}$)	% variation (RIL's)	Source of resistance
ACA-CAC_{170}	7	32 % ($P < 0.000$)	46 % ($P < 0.000$)	CDC Chico
TA76s	1	14 % ($P < 0.000$)	19 % ($P < 0.000$)	CDC Chico
TA110	7	10 % ($P < 0.004$)	13 % ($P < 0.000$)	CDC Chico
TA176	4	7 % ($P < 0.01$)	9 % ($P < 0.01$)	CDC Chico

*) The STMS markers were previously assigned into linkage groups based on the analysis of a RIL population developed from an interspecific cross between *C. arietinum* (ICC4958) and *C. reticulatum* (P.I. 489777) (Winter et al. Mol. Gen. Genet. 1999. 262:90-101)

The AFLP marker (ACA-CAC_{170}) was cloned and sequenced in order to develop a SCAR (Sequence Characterized Amplified Region) marker to simplify its use for marker-assisted selection. Interestingly, the sequence of this marker contains a tandem GA repeat (16 *n*). This SCAR marker was designated SCAR $AFAG_{140}$. The forward and reverse primer sequences of this SCAR are 5'-ttc aca aag taa tat acc tg-3' and 5'-atg agt cct gag taa cac gga-3', respectively. At 54 °C of annealing temperature these primers generated a band of 140 bp.

The utility of these markers was tested using advanced generations of breeding lines and check cultivars that were assessed for ascochyta reaction under field conditions (natural infection) in Assiniboia in the year of 2001. DNA was extracted from individual lines and scored for the presence of the SCAR and TA76s markers. The results of the analysis are presented in Table 2. Mean disease score of the lines that had both markers was 3.8 ± 0.8 which is significantly lower than the lines that had either or none of the markers present. The result demonstrated the potential of these markers for marker-assisted selection.

Table 1.2. Mean ascochyta blight score of advanced chickpea breeding lines for each combination of SCAR $AFGA_{140}$ and TA76s markers

Marker Classes	No. of lines	Disease score
SCAR $AFGA_{140}$ (A); TA76s (A)	18	3.8 ± 0.8
SCAR $AFGA_{140}$ (A); TA76s (B)	35	5.8 ± 0.7
SCAR $AFGA_{140}$ (B); TA76s (A)	26	6.2 ± 0.7
SCAR $AFGA_{140}$ (B); TA76s (B)	14	7.8 ± 0.6

The SCAR $AFAG_{140}$ and $OPH04_{880}$ markers were further used to screen a collection of elite breeding lines that were scored for reaction to ascochyta blight (Table 3). This initial test

showed that at least one cultivar (S95420) possesses the two resistance genes. Further tests to include more cultivars and breeding lines are required. Furthermore, new crosses may be made to pyramid these resistance genes.

Table 1.3. Cosegregation of markers SCAR $AFGA_{140}$ and $OPH04_{680}$ and ascochyta disease score for several chickpea cultivars

Genotype	SCAR $AFGA_{140}$	$OPH04_{680}$	Ascochyta disease score
ICCV6-3	B	A	8.3[1]
CDC Chico	A	A	2.4[1]
CDC Marengo	B	A	8.0[1]
CDC Diva	B	A	8.0[2]
CDC Xena	B	A	8.0[2]
FLIP94-48C	A	A	2.7[1]
P1279-2-1	B	B	2.3[1]
S95420	A	B	2.4[1]
ICCV96029	B	A	5.5[1]
Evans	B	A	8.0[2]
Sanford	B	A	8.0[2]

A = marker is present; B = marker is absent

[1] scores based on growth room or greenhouse conditions

[2] scores based on field conditions (natural infection) at Assiniboia in 2001

Lentil

1. Cross Indianhead x Eston

Indianhead is a black-seeded lentil with resistance to ascochyta blight; Eston is a small green lentil, susceptible to ascochyta blight. Following is the summary of the technical aspects and accomplishment for this cross. Sixty F_2 plants were developed from a cross between Indianhead and Eston. A single recessive gene, *ral2*, determines the resistance to ascochyta in Indianhead. The F_2 plants segregated for 14 resistant, 34 heterozygous (segregating) and 12 susceptible. A total of 800 RAPD primers were screened for polymorphisms between the resistance and susceptible bulks. Two primers (UBC227 and OPD10) generated DNA fragments that co-segregated with the resistance gene. The two markers ($UBC227_{1290}$ and $OPD10_{870}$) flanked the resistance gene at a distance of 12 cM and 16 cM, respectively. These markers were then converted into SCAR markers. The SCAR marker that was developed from the $UBC227_{1290}$ was lost its polymorphism between the two parents and among the F_2 plants. The SCAR marker developed from $OPD10_{870}$, however, retained its polymorphism.

2. Cross ILL5588 x L692-16-1

A RIL population that was developed at ICARDA from a cross between ILL5588 and L692-16-1 was used in the current study. ILL 5588 is one of the most important donor lines for ascochyta resistance in both Australian and Canadian lentil breeding programs. The lines were tested for their reaction to two isolates (RM 389-1 and PC-1) of *A. lentis* in a growth chamber. The isolates were chosen based on the differential reaction of the two parents to the disease.

The scoring was done at 14 days after inoculation on a scale of 0 (no symptoms) to 7 (plants are dead).

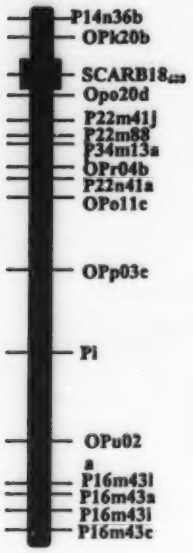
DNA from the two parents and the RI lines was extracted following the CTAB procedure. A total of 818 RAPD and ISSR primers were screened for polymorphisms between the two parents and the resistance and susceptible bulks. Primers that produce polymorphic bands between parents and the two bulks were tested on the individual RI lines. The marker data generated from this analysis were integrated into the existing linkage map developed from the same population (Eujayl et al. 1998. Theor. Appl. Genet. 97:83-89) using Mapmaker/Exp program version 3.0b. QTL analysis for the association between marker loci and the reaction to two isolates was done using QTL Cartographer program Version 1.13.

Of the 818 RAPD and ISSR primers that were screened, only 5 primers resulted in polymorphic bands between the two parents and the R and S bulks. QTL analysis indicated that only two marker loci generated using RAPD primers OPB18 and OPV1 were associated (LOD > 3.0) with the reaction to ascochyta blight. These two QTL ($OPB18_{680}$ and $OPV1_{800}$) were located on LG 2 and LG 4 (Fig. 1) of the lentil map and explained 36 % and 29 %, respectively, of the disease reaction variability (Table 4). The $OPB18_{680}$ marker is identical to RB18 marker that was previously identified from the cultivar Northfield (ILL 5588) by the Australian group (Ford et al. 1999. Theor. Appl. Genet. 98:93-98). The $OPB18_{680}$ marker was sequenced and converted into a SCAR marker to simplify the analysis (Fig. 2). The forward and reverse primer sequences for this SCAR marker are following: 5'-cca cag cag tca caa acc cta tat ga-3· and 5· -cca cag cag tat tat tat caa tat ttt g-3· , respectively. At a 60ºC annealing temperature, these primers will amplify a single band at a size of 680 bp.

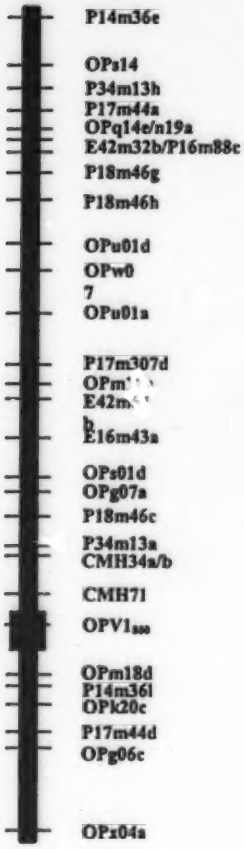
Table 1.4 Summary of QTL for reaction to ascochyta blight in a RI population of lentil derived from a cross between ILL5588 and L692-16-1

Linkage group	Closest marker to the LOD peak	LOD value	% variation	Additive effect	Source of resistance
2	SCAR $B18_{680}$	8.0	0.36 (P <0.000)	-0.84	ILL5588
4	$OPV1_{800}$	8.3	0.29 (P <0.000)	-1.06	ILL5588

To determine whether the cultivar ILL5588 contains DNA fragments similar to known resistance genes, a PCR-based approach to isolate resistance gene analogues (RGA) sequences from the lentil genome was employed. This approach relies on the use of degenerate primers, which were designed based on the conserved peptide motifs of cloned resistance genes from arabidopsis, flax and tobacco to amplify leucine rich repeats (LRR), nucleotide binding-sites (NBS) and kinase domain-containing sequences from lentil cultivar ILL5588. More than 48 RGA fragments (sizes range from 400 to 700 bp) that were generated from the amplification of lentil ILL5588 were cloned and sequenced. Sequence analysis indicated several of these genomic fragments were homologous to each other (64 to 92 % identity). These sequences will be deposited into the Genebank database at the National Center for Biotechnology Information at Bethesda, Maryland. Database search resulted some significant sequence similarities to known resistance genes, such as *RPS2* (from Arabidopsis) and *L6* (from flax). These clones were then used to probe the parental DNA's in order to determine their location on the linkage map and their association with the resistance to ascochyta. However, no polymorphism was found after hybridization of the RGA clones with the ILL5588 and L692-16-1DNA's that were cut by ten different restriction enzymes (five and six cutter). Further analysis to include new restriction enzymes is required.



LG2 (114.6 cM)



LG4 (

251.5 cM)

Fig.1.1 Integration of SCAR $B18_{680}$ and $OPV1_{800}$ on LG2 and LG4 of lentil map

Fig 1.2 Co-segregation of the SCAR $B18_{680}$ marker and the ascochyta disease reaction using the RM389-1 isolate in the RIL's

A new cross between CDC Robin x 964a-46 was made in the summer of year 2000 in order to pyramid different sources of resistance to ascochyta. CDC Robin has ascochyta resistance derived from Indianhead (recessive gene) while 964a-46 carries the ILL 5588 source of resistance. CDC Robin is also resistant to anthracnose (*race* 1). The gene for resistance to ascochyta blight in Indianhead has been tagged by flanked RAPD markers ($UBC227_{1290}$ and $OPD10_{870}$). The 198 F_2 plants from this cross segregated 146:52 (presence:absence) for the SCAR $B18_{680}$ and 132:66 (presence:absence) for the $UBC227_{1290}$, following a ratio of 3:1 (P > 0.10). These results indicated that the population segregates for the two genes. These results will be verified in the RIL's ($F_{6:7}$) which will be available in the spring of 2001. Screening for the *Ascochyta lentis* isolates that will differentiate the two sources of resistance is currently underway. Future work will include screening of the RILs for the reaction to ascochyta and genotyping using the two markers to follow the progenies that carry the two genes and facilitate the transfer of the two resistance genes into elite varieties.

1.4 Indications for Further Development – Sub-Project 1

The results of this project will be used in further investigation of the genetic structure of the ascochyta resistance gene complexes in both lentil and chickpea. Now that the pulse research laboratory is equipped with efficient extraction and analysis systems through a major infrastructure grant from CFI, we will be able to (1) develop additional markers, (2) develop low-cost genetic maps and (3) eventually use the markers in breeding applications. Through collaborations with the Australian lentil research group, and with additional funding from Saskatchewan Pulse Growers (2001-2005) we plan to expand the potential use of markers to identify additional genes for ascochyta resistance, including those from wild species of lentil.

1.5 Information on Deliverables and Performance Indicators – Sub-Project 1

The lentil breeding program has developed elite breeding lines such as 1066-E-4 and 1048-8R which were supported for registration at the PRRCG meetings in Winnipeg, 2002. Based on pedigree analysis and disease assessment, we assume that these lines carry both the ILL 5588 and Indianhead ascochyta resistance genes. We should be able to implement the marker technology in the near future to detect which pre-breeder sub-lines actually have maintained both resistance genes. Our eventual goal will be to use the markers to detect presence or absence of the genes in three-way cross F1 plants in similar applications to those used in our *Phaseolus* breeding program.

Research publications and presentations developed through this project include the following. In all publications and presentations, AFIF was acknowledged as a major contributor to the research.

M. Chowdhury, A.E. Slinkard, A. Vandenberg and K.R. Tiwari, 1998. A RAPD Marker Ascochyta Blight Resistance in Chickpea. Pulse Crops Research Workshop, 3:104-105, University of Saskatchewan.

M. Chowdhury, T. Warkentin, S. Banniza, A. Tullu and A. Vandenberg, 2000. Evaluation of RAPD Marker Linked to Ascochyta Resistance in Chickpea, pp. 39-41. Soils and Crops 2000. Extension Division, University of Saskatchewan.

Chowdhury, M.A., C.P. Andrahennadi, A.E. Slinkard and A. Vandenberg. 2001. RAPD and SCAR markers for resistance to ascochyta blight in lentil. Euphytica 118:331-337

Tar'an, B., C. Breitkreutz, L. Buchwaldt, A. Tullu, A. Vandenberg, and T. Warkentin. 2002. Genetic Study and Molecular Markers for Ascochyta Blight Resistance in Lentil and Chickpea. Soils and Crops, 2002. Saskatoon, SK., Feb 21-22, 2002.

Tar'an, B., L. Buchwaldt, A. Vandenberg, T. Warkentin and I. Eujayl. 2001. Poster presented at North American Pulse Improvement Association/Bean Improvement Cooperative (NPIA/BIC) meeting in Fargo, North Dakota. October 28 – November 2, 2001

Tar'an, B., C. Breitkreutz, M. Chowdhury L. Buchwaldt, , A. Tullu, T. Warkentin and A. Vandenberg. 2002. Identification of molecular markers for resistance to ascochyta blight in two populations of chickpea and their usefulness for marker-assisted selection. (manuscript in preparation)

Tar'an, B., L. Buchwaldt, A. Tullu, T. Warkentin, A. Vandenberg and I. Eujayl. 2002. Integration of molecular markers linked to resistance to ascochyta blight and R-genes analogues into a linkage map of lentil. (manuscript in preparation)

2. TECHNICAL REPORT for SUB-PROJECT 2

Transformation of Lentil

Dr. A. Gulati, Dr. A. McHughen, Dr. P. Hayes-Schreyer

2.1 Summary of Results and Background and Objectives – Sub-Project 2

Advances in molecular genetics and biotechnology have made plant transformation and regeneration an available and powerful tool for crop improvement. The development of an efficient transformation system for the introduction of novel genes and development of fertile, genetically stable plants is lacking but essential for these advances to be applied to lentil.

Lentil shoots have been regenerated from shoot apices (Bajaj and Dhanju, 1979; Polanco et al., 1988; Singh and Raghuvanshi, 1989; Williams and McHughen, 1986); nodal explants (Ahmad et al., 1997; Polanco et al., 1988; Singh and Raghuvanshi, 1989); intact seedlings (Malik and Saxena, 1992) and cotyledonary nodes (Warkentin and McHughen, 1993). However, the major limiting factor in the establishment of a successful plant regeneration system from *in vitro* regenerated shoots for lentil is the low frequency of rooting. There are only a few reports of rooting from regenerated shoots in lentil, and the frequency of rooting is very low. Therefore, an efficient method for successful rooting in regenerated shoots is required to establish a viable system for *in vitro* propagation in lentil.

The objectives of this project were: to develop a system for rooting of *in vitro* regenerated shoots to overcome the low efficiency currently obtained with *in vitro* rooting and to develop an efficient transformation method for introducing foreign genes into lentil and to produce stable and fertile transgenic lentil plants.

In summary, transformation protocols were successfully developed by optimizing procedures developed for transformation of other crop species.

2.2 Investigation of *IN VITRO* Regeneration of Shoots and Rooting – Sub-Project 2

2.2.1 Materials and Methods

Rooting studies were conducted by using micrografting techniques on three lentil cultivars, Laird (large seed), CDC Crimson (small seed) and CDC 599-23 (small seed). A detailed study to determine effect of BA (6-benzylaminopurine) on micrografting was conducted with Laird. Seeds were sterilized and placed aseptically on ½-strength MS (Murashige and Skoog, 1962) medium solidified with 0.4% agar in Petri plates for germination at 22°C in the dark.

Cotyledonary nodes were excised from 3-d-old seedlings by removing the shoot apex and root meristem and cultured by embedding the hypocotyl end onto MS medium containing 3% sucrose and 0.7% agar, with or without BA (4.4 μM, 8.8 μM, 13.2 μM, 17.6 μM) for shoot regeneration. After 4 wk, only regenerating explants were transferred to MS medium with 4.4 μM BA and 5.8 μM GA_3 (gibberellic acid)(shoot elongation medium, SEM).

Twenty-five shoots (2-4 cm) from each BA treatment were excised, reduced in length to 1-1.5 cm and grafted onto seedling rootstocks of lentil obtained from seedlings germinated for 5-6 d in the dark at 22°C. The grafted plantlet was cultured on ½-MS medium solidified with 0.6% agar for 7-12 days. Any buds emerging from the cotyledonary node axils of the rootstock were removed. The successfully grafted shoots (15 from each BA treatment) were transplanted to pots in Redi-earth™ in a growth cabinet with 60% humidity and hardened off by covering with a glass beaker for 2-3 d. The beaker was increasingly propped up for 2 d before it was removed.

The cultivar effect was tested using the best regeneration system developed from Laird. Micrografting was tested by grafting shoots regenerated from cotyledonary nodes of all three cultivars on MS media with 8.8 μM BA either on their respective rootstocks or on Laird.

All the media were adjusted to pH 5.8 before autoclaving at 121°C and 105 kPa for 15 min. All the cultures were maintained in a growth cabinet at 22°C with 60% humidity under a 16-h cool white fluorescent light, 55 μmol m^{-2} s^{-1}. Data on cultures differentiating shoot buds or callus were scored after 4 wks and regenerating shoots were scored after 8 wks. The standard errors of the mean from three replicate experiments were calculated.

2.2.2 Results

2.2.2.1 Regeneration of shoots

'Laird' cotyledonary node explants on MS medium without BA produced 1.4 shoots/explant from the nodal area and roots from the basal embedded end (Table 2.1). The addition of BA to MS medium induced shoot bud differentiation from the axils of the cotyledonary node and callus at the basal end within 4 wks. Multiple buds regenerated on BA were transferred to S.E.M. and developed into shoots over a 4-wk period. BA at 8.8 μM induced the maximum number of shoots (4.3 shoots/explant) within 8 weeks in 80% of cultures (Table 2.1). Cotyledonary nodes were still producing new buds/shoots when these experiments were terminated after 8 wks.

Table 2.1. Regeneration and micrografting response of shoots regenerated from cotyledonary node explants of lentil cv. Laird on MS medium with BA.

Mean % of cultures	*Mean no. of shoots*[b]	*% of successful*

BA µM	Mean % of cultures		Mean no. of shoots[b]		% of successful	
BA µM	Differentiating shoot buds/callus[a]	Regenerating shoots[b]	Regenerating per explant	Micrografted	Micrografts[c]	Transplants[d]
0	0.7 ± 0.70 (1/141)	5 (1/8)	1.0	25	92.0 (23/25)	93.3 (14/15)
4.4	37.5 ± 2.77 (75/200)	70.6 ± 8.02 (53/75)	1.9 ± 0.13	25	92.0 (23/25)	93.3 (14/15)
8.8	42.6 ± 2.70 (81/190)	80.2 ± 6.16 (65/81)	4.3 ±0.24	25	96.0 (24/25)	100 (15/15)
13.2	16.8 ± 1.19 (30/178)	56.6 ± 3.75 (17/30)	2.4 ± 0.25	25	84.0 (21/25)	93.3 (14/15)
17.6	13.3 ± 1.69 (26/195)	42.3 ± 5.63 (11/26)	1.6 ± 0.20	25	92.0 (23/25)	93.3 (14/15)

Values are mean ± S.E
[a] At 4 weeks of culture (n = 141-200)
[b] At 8 weeks of culture (n = 26-118)
[c] After 12 d of grafting (n = 25)
[d] After 10 d of transplantation (n = 15)

2.2.2.2 Micrografting

Regenerated shoots grafted onto seedling rootstocks (Fig. 2.1A, B) started growing within 7-12 d. Out of 125 grafts, 84 - 96% were successful (Table 2.1). Out of 75 grafted plants, 93 – 100% survived transplantation and were grown to maturity (Fig. 2.1C, D)(Table 2.1). The grafted plants were phenotypically normal and produced normal pods and viable seeds.

Our micrografting protocol is independent of the concentration of BA used in the shoot regeneration medium as shown in Table 2.1. In addition, multiple shoots regenerated from cotyledonary node explants on BA (8.8 µM) from all three lentil cultivars were grafted to their respective rootstocks. No significant differences in the success rate of micrografting among the three cultivars and in the survival rate following their subsequent transplantation were observed (Table 2.2).

Table 2.2. Micrografting of *in vitro* regenerated shoots obtained from different cultivars of lentil. Culture medium: MS basal medium + 8.8 µM BA.

Cultivar	% cultures regenerating shoots[a]	Mean no. of shoots[a]		% successful	
		Regenerating per explant	Micrografted	Micrografts[b]	Transplants[c]
Laird	95.5 ± 2.22 (43/45)	3.52 ± 0.22	20	100.0 (20/20)	100.0 (15/15)

CDC Crimson	91.1 ± 4.46 (41/45)	3.7 ± 0.31	20	90.0 (18/20)	93.3 (14/15)
CDC 599-23	91.1 ± 3.89 (53/58)	1.9 ± 0.22	20	90.0 (18/20)	93.3 (14/15)

Values in parentheses represent ratio of explants showing response.
Values are mean ± S.E
[a] At 8 wks of culture (n =45-58)
[b] At 12 d after grafting (n = 20)
[c] At 10 d after transplantation (n = 15)

Grafting of the shoots is independent of the exposure time of the shoots to cytokinin in the regeneration medium. Success rate of micrografting of shoots, regenerated on medium with BA after 8 weeks, ranges from 83% to 96% (Table 2.1). Grafting eliminated the need for any pre- or post- treatment of the regenerated shoots with auxin for root induction as the regenerated shoots were grafted directly on the seedling rootstocks of lentil.

2.2.2.3 Transplantation

Transplantation of regenerated grain legume plantlets to soil in pots typically has a very low success rate. Generally high mortality rates are due to soft, weak stems and roots without good differentiation of vessels that were unable to acquire the nutrients from soil when transferred from agar medium. In addition, the callus formed between root and shoot and any damage to roots during transplantation increases the risks of soil borne diseases, which reduces the plantlet survival transfer in soil. In our study, the success rate of transplantation is very high, up to 100% (Table 2.1, 2.2). Grafting of shoots reduced the time from rooting to transplantation. The total time period from grafting of lentil shoots to successful transplantation is less than 2 weeks.

2.2.3. Conclusions

Micrografting of lentil shoots overcomes many rooting problems and is independent of growth regulator treatment applied to the regenerated shoots and cultivar. This technique enables the regeneration of whole plants. This will dramatically increase the production of lentil regenerants, including putative transformed plants. The micrografting of shoots results in both a significant reduction in the regeneration period and a significant increase in the production of lentil regenerants.

I2.4 TRANSFORMATION OF LENTIL

There is limited data in the literature on lentil engineering using: *Agrobacterium tumefaciens* (Halbach et al., 1998; Lurquin et al., 1998; Warkentin and McHughen, 1991, 1992, 1993); protoplast (Maccarrone et al, 1992); electroporation (Chowrira et al., 1996; Maccarrone et al., 1995) and particle bombardment (Öktem et al., 1999). To date, there are two studies which claim to have produced transgenic lentil plants, Chowrira et al. (1996) via *in planta* electroporation on nodal meristems and Barton et al. (1997) via *Agrobacterium tumefaciens*. However, neither publication provided supporting data.

2.4.1 PARTICLE BOMBARDMENT-MEDIATED GENE TRANSFER

Although there are no substantiated claims of transformation by particle bombardment in lentil there are examples in other grain legumes including: *Arachis hypogaea* (Brar et al., 1994); *Cicer arietinum* (Kar et al., 1997); *Glycine max* (Aragão et al., 2000; Christou and McCabe, 1992; Santarem and Finer, 1999); and *Phaseolus vulgaris* (Russell et al., 1993; Aragão et al., 1996). Therefore, our objective was to develop a transformation method for introducing foreign genes into lentil and the production of stable and fertile transgenic lentil plants.

In the present study two genes, acetolactate synthase (*ALS*) and hygromycin phosphotransferase (*HPT*), were used for particle bombardment mediated gene transfer. The detailed study was done using an *ALS* gene from tobacco.

The use of an acetolactate synthase mutant for the development of our protocol was based on the mode of action of the gene. Sulfonylureas and imidazolinone herbicides act by interrupting the normal acetohydroxyacid synthase catalysis of the initial step in the biosynthesis of valine, leucine and isoleucine (Lee et al., 1988). The advantage of this method over other available herbicide tolerance systems, i.e. Basta, is that the herbicide is translocated to and acts directly on the active growing centers, not as a contact herbicide (Aragão et al., 2000).

2.4.1.1 MATERIALS AND METHODS

2.4.1.1.1. Plant material and culture of explants

Two lentil cultivars, Laird and CDC 599-23 (CDC Robin) were used in our study. The commercial cultivar Laird was used for the detailed studies to optimize the variables in the transformation protocol. CDC 599-23 was used to study cultivar effect on the optimized protocol. Seeds were surface sterilized and explants were excised as described in section 1. The cotyledonary node explants were cultured on MS-2, previously reported as the best medium for regeneration. For each bombardment, 20-25 explants were arranged in the center of a 60-mm sterile Petri plate on MS-2 medium. The plates were maintained in a growth cabinet at 22°C, 16-h photoperiod with light intensity of approximately 55 μmol m^{-2} s^{-1} for 0-6 d (preculture period) prior to bombardment. All media were adjusted to pH 5.8 before autoclaving at 121°C and 105 kPa for 20 min.

2.4.1.1.2. Plasmid

DNA was purified from *E. coli* strain *DH5α* containing a plasmid with a chimeric SuRA/SuRB Hra acetolactate synthase (ALS) gene, from tobacco (courtesy of DuPont, Wilmington, DE). that confers sulfonylurea resistance in plants. The chimeric gene was constructed by inserting a fragment of the mutant SuRB S4-Hra gene containing the two mutations into the mutant SuRA C3 gene (Lee et al., 1988) producing a chimeric gene of 4637 base pairs including the endogenous tobacco SuRA C3 promoter and terminator. The plasmid was constructed by inserting chimeric SuRA/SuRB Hra gene in the polycloning region between *PstI* and *SalI* of the vector pBUC 19 which contains ampicillin resistance and produced a plasmid of 8016 base

pairs. Plasmid DNA was purified from the bacterial cells using the QIAGEN Plasmid Maxi kit. The resulting DNA pellet was resuspended in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0). The DNA concentration was quantified using an ultraviolet spectrophotometer (GeneQuant II RNA/DNA calculator. Pharmacia Biotech) at 260 nm and 280 nm and adjusted to 1.0 μg/μL for bombardment experiments.

2.4.1.1.3 Particle Bombardment

For bombardment, 5.0 μL of plasmid DNA (1.0 μg/μL) was added to a vigorously vortexing 50 μL aliquot of sterilized gold particles (1.0 μm) suspended in a sterile solution of 50% glycerol and was precipitated onto the particles by $CaCl_2$ – spermidine (Klein et al., 1987). After final washes in 80% and absolute ethanol, the DNA-gold particle mix was resuspended in 60 – 70 μL of absolute ethanol. Ten μL of the DNA-coated gold suspension was pipetted and spread onto the center of each macrocarrier. The loaded macrocarriers were placed inside a container with drying material (Dryrite™ (anhydrous calcium sulfate), W.A. Hammond Drierite Col. Xenia, Ohio) and all the loaded macrocarriers were used within 30 min of loading.

The precultured and non-precultured cotyledonary nodes cultured on MS-2 media were bombarded once with DNA-coated gold particles using a PDS 1000/He Delivery system (Biorad Laboratories, Ontario, Canada) with a vacuum pressure of 66 cm of mercury. The microcarrier launch assembly was in the second position from the top, the macrocarrier travel distance was 6 mm and the samples were placed three positions down. Factors affecting transformation efficiency, including rupture pressure (650-1800 psi) and explant preculture period (0-6 d), were evaluated. After bombardment, the Petri plates were sealed with Parafilm® and placed in a growth cabinet under preculture conditions.

2.4.1.1.4. Selection and regeneration of putative transformants

Explants were regenerated using the best cotyledonary node regeneration protocol from section 1 with the following modifications. Bombarded explants were grown without selection for 2 d then transferred to MS-2 media with 5 nM chlorsulfuron (DuPont Nemours, USA, 98% purity). After 4 wk of culture on MS-2 with 5 nM chlorsulfuron, the growing explants were transferred to MS-1 medium (MS with 4.4 μM BAP + 5.2 μM GA_3 [gibberellic acid]) with 2.5 nM chlorsulfuron for the remainder of the culture period. The cultures were subcultured onto fresh medium with selection every 2 weeks. Data were scored 2 and 6 wk after culture on selection medium to evaluate the effect of preculture period and rupture disk pressure on survival rate and differentiation of shoots. The standard errors of the mean from two replicate experiments containing 42-60 explants each were calculated.

2.4.1.1.5. Grafting and transplantation of putative transformants

Grafting of putative transformed shoots regenerating on selection medium and transplantation of plantlets was done as described in section 1. The plants were maintained in a growth cabinet at 22°C with 16 hr day length.

2.4.1.1.6. Screening of putative transformants using herbicide painting

A subsample of twelve T_0 plants were randomly selected and subjected to herbicide leaflet-painting assay. The metsulfuron methyl herbicide, Ally®, was applied at 9.2g/ha, a concentration representing twice (2X) the recommended active field rate to a single leaflet of each plant (at 6-8 node stage), using a small paintbrush (No. 2). This treatment in control plants results in plant death within 2 wks. Results were observed 14 d after treatment. The surviving as well as non-tested T_0 plants were allowed to mature, flower, self-pollinate and set seed.

2.4.1.1.7. Progeny analysis using herbicide painting

Inheritance of the introduced transgene was analyzed using herbicide application. T_1 seeds (30-120) harvested from 6 herbicide resistant and 14 non-tested putative transformants were germinated in 6 x 5 x 6 cm pots containing Rediearth™ with growth conditions identical to those used for T_0 transformants. Once the seedlings were at 2-3 node stage, herbicide testing by leaflet painting, as described in the previous section, was carried out. The results were scored 3 weeks after application. The surviving plants were allowed to mature, flower, self-pollinate and set seed.

2.4.1.1.8. Plant DNA isolation

Genomic DNA was extracted from freeze-dried leaf tissues of T_1 putative transgenic and non-transformed lines using a Qiagen DNeasy kit. DNA was eluted in TE (containing 10mM Tris and 0.1 mM EDTA, pH 8.0).

2.4.1.1.9. PCR procedure

Polymerase chain reaction (PCR) amplification of a 835-bp DNA fragment from the mutant tobacco *ALS* gene was carried out using primer pair K1 (5'-GCTATAACCGGTCAAGTTG-3') and K2 (5'-TGGATAGCATATTGCGGAG-3'). The amplification reaction was carried out using a Perkin-Elmer Cetus, (Norwalk, CT), DNA Thermal Cycler 9600. The final PCR reaction volume was 50 µl containing 1X PCR buffer, 200 µM each of dNTP, 2.5 mM $MgCl_2$, 400 nM of each primer DNA, 30-40 ng genomic DNA and 2.5 units of *Taq* DNA polymerase (Gibco™ Invitrogen Corp, Carlsbad, CA,). DNA was amplified under the following cycle conditions using hot start: 94 °C for 3 min (denaturation), then 35 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 1 min (extension), followed by an elongation step of 72 °C (5 min) and 4 °C until recovery. The amplified products were detected under ultraviolet light after electrophoresis in 1.2% (w/v) agarose (Gibco™ Invitrogen, ultra pure)/ethidium bromide gels in 0.5X Tris-borate buffer (TBE) (45 mM Tris-borate and 1 mM EDTA).

2.4.1.1.10. Southern hybridization analysis

Genomic DNA, 5 µg/reaction, was digested with restriction endonuclease *Dra*I (Gibco BRL®), which recognizes a restriction site present within the mutant *ALS* gene from tobacco (Fig. 2.2). Other *Dra*I restriction sites were present in the plasmid but outside of the Hra region. Restriction digests were separated by electrophoresis overnight at 25 V on a 0.8% agarose gel in 0.5X TBE buffer, then transferred by alkaline transfer (Sambrook et al., 1989) to a Hybond N^+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK). The blot was hybridized with a ^{32}P-dCTP-labelled *ALS* probe prepared by excising the 4.6 kb chimeric Hra fragment from the plasmid using restriction enzymes *Sal*I and *Pst*I (Fig. 2.2) and exposed to film at –80°C. *Hind*III-digested λ-DNA was used as a molecular size standard.

2.4.1.2 RESULTS

2.4.1.2.1. Optimization of bombardment parameters

An increase in the combined survival and differentiation noted by McCown et al. (1991), Rajasekaran et al. (2000), Wijayanto and McHughen (1999) frequencies was obtained when explants were precultured on MS-2 medium before bombardment with 900 psi rupture pressure (Table 2.3). Ten percent of the explants differentiated when bombarded immediately after their excision and grown on selection medium. Preculturing for 2 to 4 d produced the maximum combined survival and differentiation of shoots frequencies (Table 2.3). The success of bombardment depends on the accurate penetration of plant tissues and transfer of DNA with minimal (i.e. non-lethal) damage to the target cells. The recovery of transformed plants depends

on many factors: the division of transformed cells, their continued growth on selection media and the maintenance of their regeneration capacity (Morrish et al., 1993). Therefore the increased production of shoots might be a result of induction of differentiation in the undifferentiated cells located at the cotyledonary node. The greater the number of actively dividing cells in the target tissues, the higher the probability of obtaining transformants. Preculture also led to an increase in the size of the explants, which might increase the target area for bombardment. A similar effect due to preculture period on plant transformation success rate has also been), and Yang et al. (1999). The explants precultured for 6 d showed differentiation of shoot primordia prior to bombardment that produced a greater proportion of escapes, defined as those shoots which died between the 2nd and 6th wk of selection *in vitro*.

Table 2.3. Effect of preculture period and rupture pressure on transformation of lentil cv. Laird cotyledonary node explants.

	Percent of explants	
	Surviving*	Differentiating shoots**
Preculture duration (d) at 900 psi		
0	25 ± 5	10 ± 6
1	20 ± 0	23 ± 13
2	53 ± 3	37 ± 7
4	50 ± 10	34 ± 10
6	45 ± 5	25 ± 5
Rupture pressure (psi) with a 2 d preculture period		
650	55± 5	5 ± 5
900	67.7± 12.3	36.1 ± 13.9
1100	41.6 ± 8.3	14.2 ± 5.8
1300	31.4 ± 11.4	5 ± 5
1550	15.5 ± 4.4	0
1800	8.9 ± 1.1	0

Values are mean ± S.E.
N= 80-110
* After 2 weeks on selection medium
** After 6 weeks on selection medium

Using a 2 d preculture period as the standard, we compared the effect of different rupture pressures (650-1800 psi) on shoot differentiation after bombardment. The highest survival and differentiation frequencies were observed with 900 psi rupture pressure. Increasing or decreasing the pressure from 900 psi had a negative effect on the survival and regeneration response of the explants (Table 2.3). Rupture pressures of 1500 and 1800 psi resulted in no shoot differentiation. The increase of rupture pressure is directly associated with the velocity of the bombarded particles and thereby affects the depth of microcarrier penetration to specific cell layers of target tissue. In grain legume cotyledonary node explants, shoots appear to originate from epidermal or subepidermal cells as reported for: *Cicer arietinum* (Murthy et al., 1996;

Polisetty et al., 1997); *Glycine max* (Wright et al., 1986); and *Vigna radiata* (Gulati and Jaiwal, 1994). Therefore, the ideal depth of penetration of the DNA-coated particles in lentil was determined to be the epidermal or subepidermal cell layers. Low pressure may not be sufficient to propel gold particles into epidermal cells, and the higher pressure might have injured the cells, which later could not proliferate. Thus, a moderate rupture disk pressure of 900 psi was identified as optimal.

2.4.1.2.2. Selection of transformants

Visual examination of the explants after bombardment revealed the proliferation of shoots from the axils of cotyledonary nodes. After 2 wk on selection medium with 5 nM chlorsulfuron, shoot bud differentiation was observed. Some shoots elongated quickly, but these shoots were excised and discarded as escapes. Continuous culturing on 5 nM of selection resulted in the formation of callus with shoot buds, which failed to elongate. By reducing the selection pressure to 2.5 nM after 4 wks, shoot buds were able to elongate into shoots (Fig. 2.3A). At subsequent 2-wk transfers, shoots that showed any leaf browning and withering were identified as escapes and discarded. Only green and healthy shoots were excised and micrografted. Out of 600 explants bombarded with 900 psi rupture pressure in sets of 25-30 explants/shot, 36 putative transformants were successfully transferred to soil, resulting in a 6% transformation efficiency. In another set of experiments with 190 explants bombarded with 1100 psi rupture pressure, 6 putative transformants were transferred to soil, providing a 3.1% transformation efficiency.

2.4.1.2.3. Herbicide painting of T_0 transformants

Putative T_0 transformants were tested for herbicide tolerance by painting leaflets with 2X Ally®. Out of 12 plants tested by leaflet painting, 9 survived the test and 3 died (Fig. 2.3B,C). The death of one plant within a week of application of the herbicide indicates that it was an escape. Two of the 9 plants that survived the leaflet paint test were subsequently lost due to insect damage. The death of 2 other plants at extended intervals following application of the herbicide suggests possible chimerism or weakly expressing transformants. These two plants demonstrated intervenal chlorosis, which in time spread to the entire plant and resulted eventually in death (Fig. 2.3B). Particle delivery mediated transformation is known to produce chimeric plants (Christou, 1990). The extent to which the axillary shoot buds from the cotyledonary node explants had organized after 2 d of pre-culture likely correlates to the findings of Gulati and Jaiwal (1994) with mungbean cotyledonary node explants, where initial divisions in the epidermis and underlying subepidermal layers had already occurred. However, experiments by Warkentin (1992) showed that the impact of wounding the axillary meristem in lentil cotyledonary nodes was an increase in shoot production verses the production in non-wounded cotyledonary node explants. This implies the creation of novel meristematic zones as a wounding response. This indicates that the putative transgenic shoots produced could have had a multicellular origin influenced by the extent of necrosis and transformation in existing meristematic cells. This would further support the chimeric nature of some of the transgenic T_0 plants. Continuous selection through all the stages of culture is effective for the recovery of transformants, but there are usually some non-transformed escapes. Use of highly regenerative multicellular tissues as transformation targets also increases the possibility of obtaining escapes and chimeric plants because selection pressure is not stringent and non-transgenic tissue can proliferate in the presence of transgenic tissue. Some of the remaining 7 plants also displayed slight herbicide damage including yellow spots or intervenal yellowing on some leaflets and the death of the leaf with the painted leaflet (Fig. 2.3C). The plants were otherwise phenotypically normal and produced normal pods and viable seeds.

2.4.1.2.4. Progeny screening

Progeny analysis is important to confirm the stable integration of the introduced gene. Out of 37 genotypes that produced seeds, 20 were analyzed for segregation of the *ALS* gene by leaflet paint testing of seedlings (Table 2.4). The other 17 genotypes were tested while developing the leaflet paint test and were therefor subjected to differential selection levels and methods. Three weeks after leaflet painting with 2X Ally, the segregation for sensitive and resistant phenotypes was observed (Fig. 2.3D). The surviving plants were phenotypically normal (Fig. 2.3E).

Table 2.4 provides the segregation data analysis for the *ALS* gene in the T_1 progeny. A χ^2-test was performed on the segregation data to determine the goodness of fit to a 3 resistant : 1 sensitive ratio. Non-Mendelian inheritance in most of the T_1 progeny was observed, with a lower than expected frequency of resistant phenotypes. Our results correlate with other studies (Christou et al., 1989; Bean et al., 1997) where the T_0 chimeras for the introduced marker gene produced more non-resistant progeny than expected and resulted in non-Mendelian inheritance. Christou and McCabe (1992) proposed that this was due to the targeting of multicellular initials which produced chimeric plants that did not always produce progeny with the introduced gene. According to Aragão et al. (1996), non-Mendelian inheritance was not due to chimerism in *Phaseolus vulgaris*, since the position of the transgenic seeds were randomly distributed in the mother T_0 plant. These results might be due to some de-stabilization of the chromosome structure caused by the inserted foreign gene and a poor transgene transmission to the progeny.

Table 2.4. Segregation analysis of putative T_1 Laird transformants (N/A not applicable).

T_0 transformants	No. of T_1 seeds germinated	Germination %	After 3 weeks of leaflet paint testing		χ^2 –test for 3:1 ratio (p value)
			Resistant	Sensitive	
2	30	93.3	4	24	55.05 (0.00)
5	60	93.3	6	50	123.43 (0.00)
7	60	100	6	54	135.20 (0.00)
8	30	100	0	30	N/A
9	60	100	41	19	1.4 (0.23)
10	32	93.7	3	27	67.60 (0.00)
11	32	100	3	29	73.50 (0.00)
12	32	100	12	20	24.00 (0.00)
15	32	93.7	4	26	60.84 (0.00)
18	32	96.8	0	32	N/A
19	60	100	32	28	15.02 (0.00)
22	60	100	22	38	47.02 (0.00)
23	32	100	0	32	N/A
31*	32	71.8	7	25	48.17 (0.00)
34*	55	92.7	11	40	77.65 (0.00)
35*	32	87.5	6	26	54.00 (0.00)
36*	90	100	27	63	97.20 (0.00)
39	30	100	6	24	48.40 (0.00)
41*	30	100	16	14	7.51 (0.02)
42*	120	95.8	44	71	82.79 (0.00)

*Leaflet paint tested at T_0

2.4.1.2.5. Molecular characterization of the regenerated T_1 transformants

The progeny of 7 genotypes were screened using PCR analysis (Table 2.5, Fig. 2.4). Oligonucleotide primers specific to the *ALS* gene sequence amplified the expected 835 bp fragment from each T_1 plant tested, but no amplification was observed from control plants (Fig. 2.4). The PCR analysis showed that 78% (22/28) of the putative transformants screened were positive.

Southern hybridization analysis was performed to provide evidence of stable integration of the introduced *ALS* gene into the genome DNA of the lentil plants. The total DNA extracted from randomly chosen 28 leaflet paint test resistant T_1 plants was digested with restriction endonuclease *Dra*I (results shown only for 11 of these plants in Fig. 2.5). Digestion of chimeric Hra fragment in the plasmid using this enzyme generated two fragments approximately 3.8 kb and 2.3 kb in sizes; such fragments were obtained from all the transformants. No hybridization was seen in DNA from a non-transformed control (Fig. 2.5). The presence of the *ALS* gene fragments was observed in 20 of the 28 plants analyzed (Table 2.5). Plant 9 had two false PCR positive T_1 progeny since they produced negative southern results (Table 2.5).

Table 2.5. PCR analysis and Southern blotting for the *ALS* gene in the T_1 Laird transformants of 7 putative transgenic genotypes (events).

Genotype	Number of plants*	Number of positive plants by	
		PCR	Southern blotting
2	1	1	1
9	3	3	1
34	4	3	3
36	1	1	1
39	2	2	2
41	11	8	8
42	6	4	4
Total	28	22	20

* All plants were leaflet test positive.

To determine cultivar effect the optimized protocol for transformation of Laird was applied to lentil cultivar CDC-599-23. Three T_0 plants were tested following the protocol previously described for Laird. Two of the 3 plants showed no or little effects from the herbicide painting. They were phenotypically normal and produced normal pods and viable seed. Leaflet paint testing of 60 T_1 progeny from each T_0 plant was conducted (Table 2.6). PCR (Fig. 2.6) and Southern blot analysis (Fig. 2.7) of the PCR positive T_1 plants confirmed the presence and integration of transgene in the progeny.

Table 2.6. Segregation analysis of putative CDC 599-23 transformants in the T_1 generation.

T_0 transformants	No. of T_1 seeds germinated	Germination %	After 3 weeks of leaflet paint testing		χ^2 –test for 3:1 ratio
			Resistant	Sensitive	
51*	60	100	2	58	164.36
52*	60	100	2	58	164.36
53*	60	100	6	54	135.20

* Leaflet paint tested at T_0

2.4.1.3 Hygromycin phosphotransferase *HPT* gene

Our studies conducted with lentil utilizing NPT II as the selectable marker have indicated that it is not highly effective in lentil (see section B). To date there are no publications on the use of hygromycin phosphotransferase as a selectable marker in lentil. Due to the unavailability of DuPont's sBAD plasmid for *Agrobacterium* work, two pCAMBIA plasmids were tested using the above described particle bombardment protocol to evaluate their usability.

2.4.1.3.1. Materials and Methods

Two lentil cultivars, Laird and CDC 599-23 were used to evaluate the use of *HPT* as a selectable marker. Cotyledonary nodes (CN) excised from 3-d old aseptically grown seedlings were bombarded using β-glucuronidase (*GUS*) as the reporter and the hygromycin phosphotransferase gene (*HPT*) as the selectable marker. For shoot regeneration, the same protocol recorded previously for the *ALS* gene was used substituting hygromycin B for chlorsulfuron.

The selection pressure was determined by placing CN explants on shoot regeneration medium (SRM) containing different concentrations (0, 5, 10, 15, 20 and 25 mg/L) of hygromycin B. Explant growth was documented weekly. The plasmids pCAMBIA-1200 and pCAMBIA-1201, which carry *HPT* or *HPT* and *GUS* genes, respectively, were precipitated onto 1.0 μM gold particles for bombardment of the CN explants utilizing the same protocol described previously for the *ALS* gene. Twenty-four hours after bombardment, the explants were transferred to SRM with 10 mg/L hygromycin for two weeks. Subsequently they were transferred to fresh SRM with 5mg/L hygromycin every two weeks. All the cultures were maintained at 22°C with 16/8-hour photoperiod. At various intervals, *GUS* activity was analyzed histochemically using X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide).

After two months of culturing on selection media, DNA was extracted from the growing calli using a Qiagen DNeasy kit. The DNA extractions were analyzed using PCR, following the same protocol reported the for *ALS* gene with *HPT* primers (P_1- 5' CGAAATTGCCGTCAACCAAG 3', P_2- 5' GAACTCACCGCGACGTCTGT 3') and *GUS* primers (P_1- 5' TTTAACTATGCCG-GGATCCATCGC 3' , P_2- 5' CCAGTCGAGCATCTCTTCAGCGTA 3') which produce a 837 bp and 530 bp fragment respectively. DNA from non-bombarded calli was used as negative controls for PCR analysis.

2.4.1.3.2 Results

The CN explants on the six concentrations of hygromycin showed a decrease in callus growth and an inhibition in shoot regeneration, which correlated with an increase in the concentration of hygromycin in the medium. On control medium (no hygromycin) 70% of explants were able to regenerate shoots. At 5 mg/L, only 20% of the explants were able to produce callus whereas at 10 mg/L, 5% of the explants showed callus formation, which died within one month of culture. Complete inhibition was observed at 15 mg/L and higher concentrations. No shoot regeneration was observed on any media supplemented with hygromycin.

Transformation experiments were conducted using the plasmids pCAMBIA-1200 (Fig. 2.8A) and pCAMBIA-1201 (Fig. 2.8B). Explants were transferred 24 hours after bombardment to medium containing 10 mg/L hygromycin and cultured for 14 days after which the hygromycin was reduced to 5 mg/L. Inhibition of growth and tissue necrosis was observed on the majority of the explants after 3 weeks on 5 mg/L hygromycin (Fig. 2.9A). However, a few explants grew and produced calli within 2 months of culture on 5 mg/L hygromycin selection medium (Fig. 2.9B).

The effect of preculture of explants on SRM for 0, 24, 48, 72 and 120 hours was studied to determine the exact stage of explants for bombardment. A preculture period of 24 - 48 hours was found the best for bombardment as 18 - 20% explants showed survival after one month of culture on selection medium.

Transient GUS expression was confirmed histochemically 24 - 48 hours after bombardment with sample explants from experiments bombarded with pCAMBIA-1201. GUS expression was found to negatively correlate with increasing time elapsed, and no expression was detected 10 days after bombardment. Untested surviving explants from pCAMBIA-1201 and all surviving explants from pCAMBIA-1200 were transferred to selection medium biweekly.

PCR analyses were performed on 12 randomly selected surviving calli from both plasmids after more than 2 months on selection media. The expected *HPT* (837 bp) fragment was detected in one callus line transformed with pCAMBIA-1200 (Fig. 2.10A). A positive PCR fragment (530bp) for GUS was obtained in one callus line with pCAMBIA-1201 (Fig. 2.10B).

2.4.1.4. CONCLUSIONS

Hygromycin phosphotransferase (*HPT*) can be used as a selectable marker for lentil transformation studies. Further work is required to determine the best selection levels for hygromycin to be effectively used as a selection agent.

Our study provides a method for particle bombardment based gene transfer in lentil using highly regenerable cotyledonary node meristems capable of producing transformants independent of callus phase. The system uses rapid selection coupled with micrografting and plantlet identification allowing the reproducible production of phenotypically normal, fertile transformants which transmit a functional transgene to the progeny. The bombardment protocol reported here provides a genetic transformation system for lentil.

2.4.2 *Agrobacterium tumefaciens*-MEDIATED GENE TRANSFER

Poor rooting is a major problem in the studies in the literature on lentil transformation via *Agrobacterium tumefaciens*. Production of transgenic shoots are recorded by Halbach et al., (1998), Lurquin et al., (1998) and Warkentin and McHughen (1992, 1993), but no transgenic plants were obtained. Barton et al. (1997) have claimed to have produced T_1 Basta resistant (*pat* gene) transgenic lentil plants through *Agrobacterium tumefaciens*-mediated gene transfer, however, no technical details have ever been published.

2.4.2.1. Materials and Methods

Cotyledonary node (CN), shoot tip and embryonic axes explants of lentil cvs Laird, CDC 599-23 and CDC Crimson excised from 2-d-old aseptically grown seedlings. The explants were inoculated with an *Agrobacterium tumefaciens* harboring a plasmid with a ß-glucuronidase gene (*GUS*) with a 35s promoter as the reporter and neomycin-phosphotransferase (nos-*NPTII*-nos) as the selectable marker.

The selection level for kanamycin was determined by growing the explants on shoot regeneration medium (SRM) containing different concentrations (0, 50, 100, 200 mg/L).

The explants of all three lentil cultivars were prepared following the regeneration protocol previously described. Inoculations (5-60 minutes) with an overnight culture of the *Agrobacterium* (OD 660: $1x10^9$-$10x10^9$) were done. The explants were rinsed in sterile distilled water 3-4 times before being placed on sterile filter paper on SRM. After a co-cultivation period (1-6 days) the explants were transferred to SRM supplemented with 50 mg/L kanamycin. The growing explants were tested for GUS expression (1-21 days) after inoculation. Seven days after inoculation, the growing explants were transferred to fresh SRM with selection every two weeks.

2.4.2.2. Results

Explants cultured at 100 mg/L and 200 mg/L turned brown within 7 days. Explants cultured at 50 mg/L were still green but displayed no bud development at 3 weeks. On control media 80-85% of the cotyledonary node and the shoot tip explants produced shoots, within 3 weeks. 60-65% of the embryonic slices explants produced shoots within 4 weeks.

GUS expression was observed in 30% of the explants, but it was not stable in explants inoculated with and selected on 50 mg/l kanamycin for 30 – 45 days. All the explants died after 45 days of culture.

2.4.2.3. Conclusion

Neomycin-phosphotransferase gene (nos-*NPTII*-nos) is poorly expressed in lentil and therefore a poor selectable marker.

2.5 FIELD TESTING OF TRANSFORMED T_2 LENTIL

The confirmation of stabile transformed T_1 lentil, through leaflet painting, PCR and Southern analysis, identified twenty Laird and three CDC 599-23 separate transgenic events. A field trial was set up to test the expression of the transgene. The field trial was run at the University of Saskatchewan Plant Science Department's Kernen Farm Plots during the summer of 2001.

2.5.1. Materials and Methods

Three conditions were required for a transgenic plant line to be selected for the field trial, they had produced a positive PCR and Southern result and over 1 gram of viable mature seed were available. Seventy-six lines from 13 Laird transformation events and 6 CDC 599-23 from 2 transformation events met these criteria. Based on space available and seed maturity times 1.0 - 2.5 grams of seed from 40 lines representing 8 Laird transformation events and 1.0 grams - 1.5 grams of seed from 6 lines representing 2 CDC 599-23 different transgenic events were seeded by hand in rows 1 foot apart May 15.

Seed were scarified and pretreated with Crown® at the recommended rate before planting. The field plot was monitored weekly until harvest. These plants were sprayed with a small plot sprayer at the 6-8 node stage with Ally® at 2X the recommended active field rate (see ALS section on leaflet painting). The root system of all plants that were lost after reaching the anthesis stage were examined, as were the roots of all plants at harvest. All ripe pods were hand picked before dehiscence of the pod to prevent loss of the T_3 seed.

2.5.2. Results

Germination rates were in the 60-80% range for all lines. After spraying, an extended period with below rainfall resulted in a severe environmental stress that combined with the herbicide effect. Strong winds and very low rainfall resulted in many plant tops being twisted off.

Viable seed was collected from 20 of the 40 Laird seed lines tested and 2 of the 6 CDC 599-23 seed lines tested (Table 2.7). The 20 Laird seed lines came from 6 different transgenic events and the 2 CDC 599-23 seed lines came from 1 transgenic event.
A complete lack of nitrogen fixing nodules was observed on all plants. This result in a substantial decrease in yield (Saskatchewan Pulse Growers, 2000), without the additional stress of a herbicide(Saskatchewan Pulse Growers, 2000). treatment.

Fig. 2.7. 2001 Field trial yields for T2 transformants

Parental Cultivar	Seed source Number	Seed Line ($T_0 + T_1 + T_2$ number) (grams)	Yield
Laird	13685-2-1	2-2-1	0.3
Laird	13685-2-2	2-2-2	0.5
Laird	13686-3-1	5-3-1	0.2
Laird	13688-1-1	9-1-1	0.6
Laird	13689-4-1	34-4-1	0.3
Laird	13689-4-2	34-4-2	1.4
Laird	13689-5-1	34-5-1	2.5
Laird	13689-5-2	34-5-2	0.2
Laird	13890-1-1	41-1-1	0.2
Laird	13890-3-1	41-3-1	0.8
Laird	13890-4-1	41-4-1	4.9

Laird	13890-4-2	41-4-2	0.5
Laird	13890-4-3	41-4-3	2.8
Laird	13890-4-4	41-4-4	0.2
Laird	13890-4-5	41-4-5	3.2
Laird	13890-6-1	41-6-1	0.5
Laird	13890-6-2	41-6-2	1
Laird	13890-6-3	41-6-3	0.6
Laird	13890-7-1	41-7-1	0.3
Laird	13891-4-1	42-4-1	0.5
CDC 599-23	13893-4-1	5351-4-1	0.9
CDC 599-23	13893-4-2	5351-4-2	1.5

2.5.3. Conclustions

The survival and yield of field plants during the 2001 crop season were compromised by severe drought conditions. 20 Laird lines from 6 transgenic events and 2 CDC 599-23 lines from 1 transgenic event produced viable seeds. However due to severe drought conditions and the lack of nitrogen fixing nodules on all investigated plants the results for the plants that did not produce seed are inconclusive.

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2.7 Indications for Further Development – Sub-Project 2

The complex issue of the acceptance of the use of genetic modification in pulse crops remains unresolved. At this time, further commercialization of this technology in lentil genetic improvement will not proceed until clear marketplace signals are received.

2.8 Information on Deliverables and Performance Indicators – Sub-Project 2

Hygromycin phosphotransferase (*HPT*) can be used as a selectable marker for lentil transformation studies. Further work is required to determine the best selection levels for hygromycin to be effectively used as a selection agent. Neomycin-phosphotransferase gene (nos-*NPTII*-nos) is poorly expressed in lentil and therefore a poor selectable marker.

Improved methods for transforming lentil using particle bombardment were developed during this project. Our study provides a method for particle bombardment based gene transfer in lentil using highly regenerable cotyledonary node meristems capable of producing transformants independent of callus phase. The system uses rapid selection coupled with micrografting and plantlet identification allowing the reproducible production of phenotypically normal, fertile transformants which transmit a functional transgene to the progeny. The bombardment protocol reported here provides a genetic transformation system for lentil.

Research publications of these results are currently in manuscript form, but will not be published until issues related to intellectual property of the information are resolved.

3- TECHNICAL REPORT for SUB-PROJECT 6

Development of Molecular Markers for Anthracnose Resistance in Lentil
Dr. A. Tullu, Dr. L. Buchwaldt and Dr. A. Vandenberg

3.1 Summary – Sub-Project 6

Anthracnose is one of the major diseases affecting lentil production in western Canada. Few anthracnose resistant lines have been identified in the breeding program. The mode of inheritance of genes for anthracnose has not been throrougly investigated. The objective was to study the genetics of resistance to anthracnose in germplasm line PI 320937. Recombinant inbred lines were been developed using a cross between Eston and PI 320937. They were infected with one of the most prevalent isolates of *Colletotrichum truncatum*, 95B36. Results indicated that one dominant gene controls resistance to anthracnose and it was designated as

LCt-2. The level of resistance is modified by the minor genes. Whether the gene(s) in PI 320937 is the same as that of other resistant lines is yet to be determined.

Additional research was conducted to identify markers linked to the gene for resistance in recombinant inbred lines developed from a cross of Eston (susceptible) and PI 320937 (resistant). Resistance in PI 320937 was dominant. Bulked segregant analysis was employed and two contrasting DNA bulks were constructed. The bulks were screened with 5 AFLP primer combinations and 700 RAPD primers. Two RAPD markers, $OPEO6_{1250}$ and $UBC\text{-}704_{700}$ located at 6.5 cM and 10.5 cM from *LCt-2* locus, respectively, and 3 AFLP markers were identified. The markers could be useful to tag *LC-2* locus and in marker assisted selection for resistance to anthracnose in segregating populations of lentil in which PI 320937 was used as the source of resistance.

3.2 Background – Sub-Project 6

Production of lentils in Saskatchewan is increasing annually. One of the major problems is anthracnose disease. Few resistant germplasm lines have been identified and use of these lines is underway in the breeding program. The inheritance pattern of resistance genes has not been reported and the race situation of the anthracnose pathogen is still not clear. At the moment, we have 2 known isolates of anthracnose. The genetics of host resistance to the different virulent isolates existing in the Prairies need to be addressed. Evaluation of breeding and germplasm lines under field condition is very cumbersome and time consuming. In addition, the prevalence of anthracnose and ascochyta simultaneously makes it difficult to score for resistance. Greenhouse studies using individual isolates to infect germplasm and breeding lines are essential to developing a long term breeding strategy that incorporates the use of molecular markers. Development of molecular markers linked to genes for resistance will facilitate selection. Secondly, the resistant materials based on our observations carry different genes for resistance. Identification of markers linked to the gene(s) for resistance facilitates pyramiding of these genes into an adapted cultivar.

3.3 Genetics of Anthracnose Resistance – Sub-Project 6

3.3.1 Objectives

The experimental work was designed with two specific areas of investigation.

(i) Genetics of anthracnose resistance to race 1 (isolate 95B36) in PI 320937 and other resistant lines (breeding lines and germplasm)
(ii) Genetics of anthracnose resistance to other isolates of anthracnose

A systematic experimental setup was designed to screen and identify genes for resistance to different isolates (races) of the anthracnose pathogen. F1 plants and recombinant inbred populations developed from the Eston x PI 320937 cross were infected in the greenhouse to study resistance to isolate 95B36 of anthracnose.

3.3.2 Results and Conclusions

Results of screening of RILs with isolate 95B36 of anthracnose indicated that a single dominant gene controls resistance to this isolate of anthracnose. The resistance level is modified by minor genes. Our result is based on the resistance level of the resistant parent, PI 320937 and a susceptible parent, Eston. There are other resistance sources used in the breeding program. Study of the allelic relationship of the genes from other sources requires further work. When these relationships are understood, we will transfer resistance genes to the adapted background.

Currently the race situation of the anthracnose pathogen is not determined. We know that isolate 95B36 of *Colletotrichum truncatum* can differentiate the reaction of PI 320937 and Eston lentils. The need to screen selected lentil germplasm with representative samples of *Colletotrichum truncatum* available in the Prairies has received high attention among researchers. This makes gene pyramiding efficient. The search for resistance genes in the wild germplasm is under way for isolate A8 for which the cultivated lentil lacks sufficient resistance.

3.4. Development of Molecular Markers for Anthracnose Resistance – Sub-Project 6

3.4.1 Objective

To identify molecular markers closely linked to genes for resistance in PI 3320937; to study their utility in different genetic backgrounds of lentil; and to localize the gene(s) in the *Lens* genome

3.4.2. Results and Discussion

A locale map has been developed where a resistance locus and marker loci are located. Of the 5 AFLP and 700 RAPD primers screened, fifteen amplified products that were either present in one bulk or absent in the other. Further analysis produced two flanking RAPD markers $OPEO6_{1250}$ (see Figure 3.1 below) and $UBC\text{-}704_{700}$ linked to *LCt-2* locus in repulsion (6.5 cM) and in coupling (10.5 cM), respectively. Three AFLP markers, namely; $EMCTTACA_{350}$, $EMCTAAGG_{175}$ and $EMCTTAAG_{375}$ were also identified within 36.6 cM from the resistance locus (Table 1). The markers could be useful to tag *LC-2* locus and in marker assisted selection for resistance to anthracnose in segregating populations of lentil in which PI 320937 was used as the source of resistance. The utility of this RAPD marker is also demonstrated in the cultivar 'Indianhead' that is extensively used as a resistance source in the breeding program.

RAPD and AFLP screening of the RILs will continue in search of markers more closely linked to the resistance gene. Besides, we will construct lentil genetic maps containing markers and agronomic traits, including maturity/earliness and seed color and plant height. We know that in a cross of Eston x PI320937, days taken to flower and mature are significantly different. The other important aspect of mapping is to do isozymes on our population in order to relate our map to other lentil maps already developed in other laboratories.

Table 1. Chi-square test for segregation ratios of AFLP and RAPD markers in $F_{5:6}$ RILs from a cross of Eston and PI 320937 lentil

Primers	Sequences	Segregation ratio (1:1)		χ^2 (1:1)	P
UBC 704	GGAAGGAGGG	48(+)	45(–)	0.10	0.75-0.90
OPEO6b	CCACGGGAAC	46(–)	49(+)	0.10	0.75-0.90
OPEF4	GGTGATCAGG	48(+)	47(–)	0.01	0.9-0.95
UBC 229B	CCACCCAGAG	48(–)	47(+)	0.01	0.9-0.95
OPER4	CCCGTAGCAC	38(–)	56(+)	3.45	0.05-0.10
UBC 18b	GGGCCGTTTA	45(+)	48(–)	0.10	0.75-0.90
EMCTTAAG	CTT/AAG*	47(+)	45(-)	0.04	0.75-0.90
EMCTTACA	CTT/ACA*	50(+)	42(–)	0.70	0.25-0.50
EMCTAAAG	CTA/AAG*	45(-)	48(+)	0.10	0.75-0.90

+ = presence of the band, – = absence of the band
EM = corresponds to EcoR1 and Mse1 primers while * = indicates the 3 selective sequences for the primers, respectively.

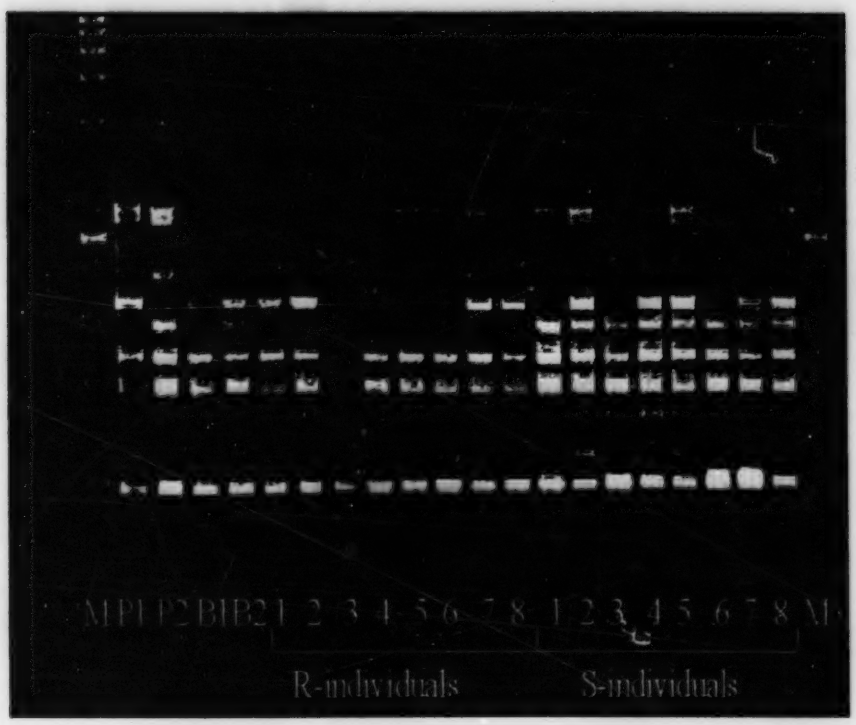


Figure 3.1. Amplification of genomic DNA using $OPEO6_{1250}$ marker. Lane M, 1kb ladder; Lane P1, resistant parent; Lane P2, susceptible parent; lane B1, resistant bulk; Lane B2, susceptible bulk; Lanes 1-8 (left), individuals in the resistant bulk; and Lanes 1-8 (right), individuals in the susceptible bulk. Arrow indicates the $OPEO6_{1250}$ band.

3.5 Indications for Further Development – Sub-Project 6

Based on the single gene inheritance scenario and the availability of markers, it seems feasible to eventually use marker assisted selection for anthracnose resistance breeding. The Saskatchewan Pulse Growers have made further financial commitment to this research area for (1) further study of the genetic relationships among alleles and genes (2) continuation of marker development (3) screening of cultivated lentil lines for additional sources of resistance (through AAFC Saskatoon – Plant Gene Resources Centre) and (4) through screening of wild species of lentil for additional sources of resistance. These areas look promising for developing a source of resistance genes and linked markers for the other main isolate of anthracnose.

3.6 Additional Information on Performance Indicators – Sub-Project 6

We are now in a position to use the available molecular markers as a tool for gathering information on the genetic structure of the breeder seed of new lentil varieties under consideration for release to the pulse industry, specifically: 1066E-4 (small green) and 1156-2-17 (medium green). Both of these show some improvement over check varieties in anthracnose response based on field and greenhouse screening tests.

3.7 Outputs and Deliverables - Publications and Presentations

Buchwaldt L., Vandenberg A., Tullu A., Bernier C.C. (2001) Genetics of resistance to anthracnose (*Colletotrichum truncatum*) in lentil. In: AEP (ed) Proceedings of the 4th European Conference on Grain Legume Research, Cracow, Poland, pp 242, Poster

Tullu, L., Buchwaldt, L., Warkentin, T. and Vandenberg, A. (2001) Inheritance of resistance to anthracnose and marker identification for resistance in lentil (*Lens culinaris* Medik.). North American Pulse Improvement Association, Fargo.

Tar'an, B., Tullu, A., Breitkreuz, C., Warkentin, T., Vandenberg, A.. and Banniza, S. (2002) Molecular Marker Techniques Applied to Pulse Crop Breeding: Assisted Selection for Disease Resistance. Pulse Days 2002 Poster.

4- TECHNICAL REPORT for SUB-PROJECT 7

Development of a Microspore Culture Program to Produce Double Haploids in Chickpea
Dr. M. Lulsdorf, Dr. J. Croser, Dr. T. Warkentin and Dr. A. Vandenberg

4.1 Summary

Double haploid technology using isolated microspore culture is a powerful tool for the induction of instant homozygosity in many crops. Developing this technology for chickpea was the objective of this sub-project. Experiments were conducted to evaluate the factors influencing androgenesis. As a result, a protocol was developed in which microspores, exposed to stress and cultured under sterile condition, could be developed into embryos. Research is in progress to accomplish the conversion of embryos into plants.

Abbreviations

ABA= abscisic acid;BAP = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; DAPI = 4,6-diamidino-2-phenylindole; GA_3 = gibberellic acid; IAA = 3-indole acetic acid; NAA = 1-naphthalene acetic acid; Picloram = 4-amino-3,5,6-trichloropicolinic acid; TDZ= N-phenyl-N'-1,2,3-thiadiazol-5-ylurea; 2,4,5-T = 2,4,5-trichlorophenoxy acetic acid

4.2 Background

Microspores are immature pollen grains that have a haploid chromosome set. Given the right conditions, pollen grains can switch from a gametophytic to a sporophytic pathway and develop into embryos and then plants. Any plants developed through this system are haploid and upon doubling, either spontaneously or by induction, are completely homozygous. The advantages of this technology are:

1. Reduction in the time required for developing new cultivars by 3-4 years. This would result in considerable savings to the breeder and to the producer, particularly if an economy of scale can eventually be used to create an efficient system. High yielding, resistant cultivars would be available more quickly. This is especially important for keeping ahead of genetic changes in the ascochyta blight fungus.
2. Easier and faster identification of recessive genes and thus potentially more efficient selection for specific traits such as ascochyta blight resistance.
3. Aiding molecular-marker assisted selection.

4. For genetic, mutagenesis, and transformation studies.

This technology is now commonly used in crops such as canola, barley and wheat. Double haploid technology has not yet been developed for any cool season food legume and generally, pulse crops are considered recalcitrant in cell and tissue culture.

4.3 Objectives

The short-term objectives were:

1. To develop a chickpea microspore culture protocol for the production of haploid embryos or calli.
2. Regenerate the microspore derived chickpea embryos into plantlets.
3. Double the chromosome number of haploid plantlets.
4. Produce seed from the doubled haploid plants.

The long-term objectives were:

1. To develop a reliable, efficient doubled haploid system for chickpea.
2. To apply this technology in the chickpea breeding program.
3. To use this methodology for other applications such as mutagenesis and transformation.

4.4 Technical Report

Androgenesis is a very complex process in which each step has to function in order to obtain results. For example, in order to screen genotypes, a medium that allows embryo development is essential. However, in order to develop a medium, responding genotypes are necessary. Furthermore, donor plant growth conditions and pre-treatments also have to be effective. If just one of the steps in this complex process is missing then the whole procedure will not yield results i.e. embryos or plants. Therefore, results obtained in some experiments were only valid under very restricted circumstances i.e. embryo development media were tested on embryos obtained from 2,4-D experiments. These media were all found to be ineffective but this conclusion is only valid for these particular 2,4-D derived embryos. Hence, even though milestones were addressed at certain times of the project, once one element of the procedure was identified, these milestones needed periodical re-evaluation.

4.4.1 Milestone: Genotype screening

A broad range of both kabuli and desi chickpea genotypes were screened for microspore culture response using different induction media (CIM-1 and CIM-4 see section 2d), with and without pre-treatments, and different donor plant growth conditions. The results were based on the number of globular embryos obtained in different experiments. Generally, the kabuli chickpea cultivars or breeding lines Garnet, Bumper, CDC Chico, CDC Diva, Yuma, B-90, 92039-5 and the desi cultivars Desiray and CDC Nika usually responded well under some or all conditions tested. There were clear cultivar (or line) interactions with several factors investigated such as pre-treatments which will be discussed in the following sections. Subsequent experiments were conducted using several of the "most responsive" cultivars.

Table 1. Chickpea cultivar screening.

Kabuli Cultivar	Response	Desi Cultivar	Response
Garnet	Good	Desiray	Good
Bumper	Good	Anna	Poor
CDC Chico	Good	Myles	Poor
CDC Diva	Good	CDC Nika	Good
Dwelley	Poor	CDC Verano	Poor
Macarena	*	DH10-31	Poor
Sanford	Poor	BS1-18	Poor
CDC Xena	Poor	DH45-1	Poor
Yuma	Good		
B-90	Good		
92039-5	Good		

* Seed germination was poor and results were probably due to the low microspore density used in the experiment rather than the genotype.

4.2 Milestone: Evaluating factors influencing microspore culture response

a) Donor plant conditions

Plants were grown in 30 cm plastic pots using Redi-earth®, with five plants per pot. Two weeks after seeding, the plants were fertilized with 75 g per pot of Nutricote® 13-13-13 N:P:K slow release fertilizer together with 3 g per pot 20-20-20 N:P:K water soluble fertilizer. Plants were given with 2 g per pot of the water-soluble fertilizer once per week.

Healthy donor plants are a pre-requisite for embryogenesis. Hence various greenhouse and growth chamber conditions were examined to determine their effect on microspore viability. The first test involved greenhouse versus growth chamber conditions. Material grown in the phytotron under controlled conditions showed excellent microspore viability. Similarly, donor plant growth was equally well in the greenhouse. However, temperature, humidity, day length, and light intensity varied considerably during the year. Plants grown under sub-optimal conditions can still be embryogenic, however, frequency of embryogenesis is usually reduced and the results are often inconsistent. Hence, all subsequent experiments were conducted using phytotron-grown material.

The second test involved the determination of optimal growing conditions in the phytotron. A humidity level of 50 to 60% was found to be best for plant growth while minimizing the risk of mold contamination in our cultures.

A third experiment involved the effect of light intensity on donor plant growth by comparing material grown in growth chambers with high sodium lights (light intensity app. 475 $\mu Es^{-1}m^{-2}$) and chambers with fluorescent tubes and incandescent bulbs (light intensity app. 400 $\mu Es^{-1}m^{-2}$). At this point, there seems to be no difference between the two light intensities. Hence, donor plants were usually grown in the fluorescent/ incandescent light chamber with a photoperiod of 16 hours.

In a fourth trial, a two-phase temperature regime was found to be optimal for plant growth: phase 1 from seedling to pre-flowering uses a day temperature of 22°C and a night temperature of 16°C, whereas in phase 2, day temperature is reduced to 19°C and the night temperature is 14°C. Plants grown under these conditions have a short juvenile period, a longer 'bud collection' period and microspores obtained from these plants are viable and responding to induction. However, since results were based on number of globular embryos obtained from different experiments and not on number of haploid plants, adjustments might have to be made once the protocol is further developed.

b) Pre-treatment

Pre-treatments are commonly necessary in order to obtain a switch from the gametophytic to the sporophytic pathway. These pre-treatments may be applied to donor plants, buds, anthers or microspores. In canola, a heat treatment of the microspores of 32.5°C for 8 hours is required whereas in barley, spikes are exposed to the combined effect of cold (3 days at 4°C) plus mannitol (0.3M).

The first test involved cold storage of buds from three different cultivars (Bumper, Desiray and Yuma) for 0, 1, 2, 4, 8, and 14 days at 4°C in the fridge. The buds were kept in sealed Petrie® dishes with a moist tissue placed under the buds to retain humidity. Results showed that response to cold treatment was genotype dependent. For cultivar Yuma, a cold period of 8 days was optimal for androgenesis whereas for cultivar Desiray, a 4-day cold period was most effective. In contrast, cold pre-treatment was not effective for cultivar Bumper.

The second pre-treatment involved placing buds for 3 days in solutions of different (0.3, 0.4, 0.5, 0.6 M) mannitol concentrations. The mannitol treatments were found to be detrimental for androgenesis.

The third test combined the effect of cold with mannitol (0.3 M) treatment. Similarly, this pre-treatment did not improve the embryogenic response.

A preliminary test was also done on heat (30°C) pre-treatment (48 hours) of microspores. Microspore viability was considerably reduced but some pro-embryo formation was observed. However, the relatively poor response could have resulted from mold contamination in the cultures. All attempts to eliminate mold were unsuccessful but this pre-treatment might be worthwhile re-examining.

c) Developmental stage of the pollen grain

All buds contained pollen in different stages of development from tetrad (Fig. 1) to mono-nucleate (Fig.2) and bi-nucleate with a dense generative and a diffuse vegetative nucleus. However, each size had a predominance of certain stages. Using a bud size of about 3.5 - 4.5 mm, the majority of microspores were in the mono-nucleate stage. In general, younger buds seem to be more responsive than older ones. Results from other crops have shown that symmetrical microspore nuclei division is the first indicator for a switch from the gametophytic to the sporophytic pathway. Similarly in chickpea, we found evidence for symmetrical nuclei division (Fig. 3).

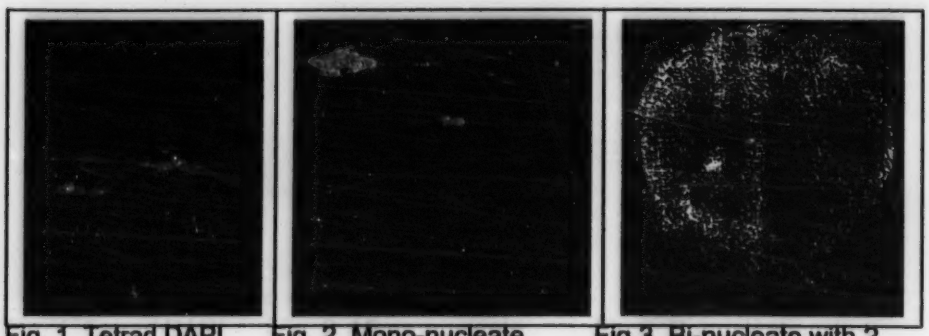
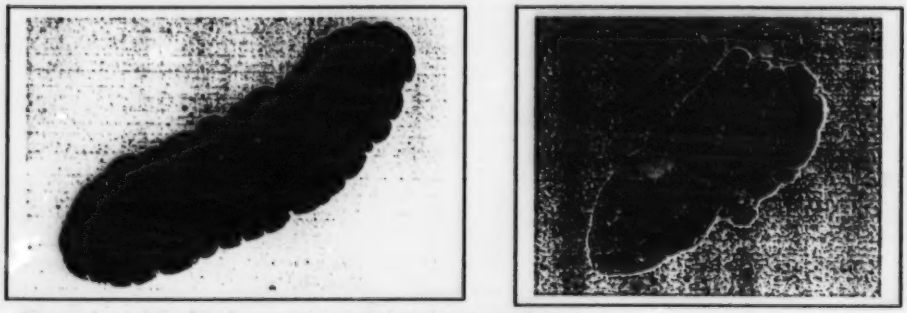


Fig. 1. Tetrad DAPI Stained. Fig. 2. Mono-nucleate Microspore. Fig.3. Bi-nucleate with 2 similar nuclei.



Figs. 4 and 5. Acetocarmine-stained pro-embryos.

Embryo development from microspores followed the usual stages of zygotic embryos from pro-embryo (Figs. 4 and 5) through globular (Figs. 6 and 7) to occasionally heart-shape stage (Fig. 8) and very rarely to torpedo-shaped (Figs. 9 and 10) stage.

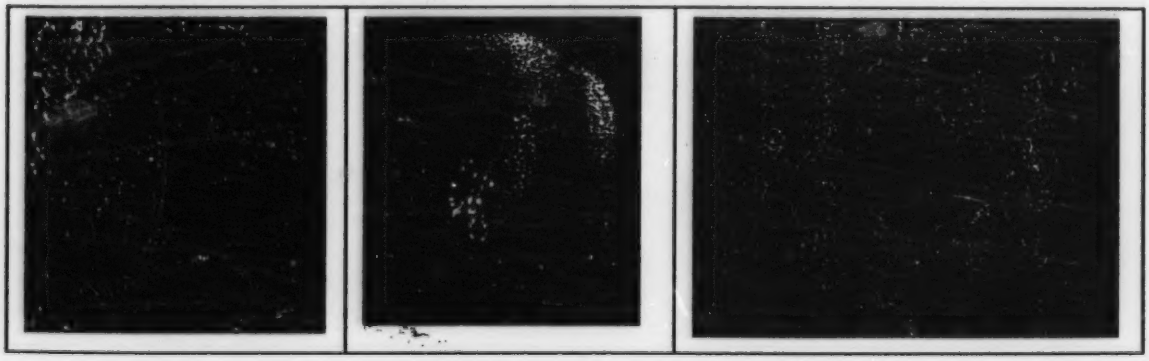
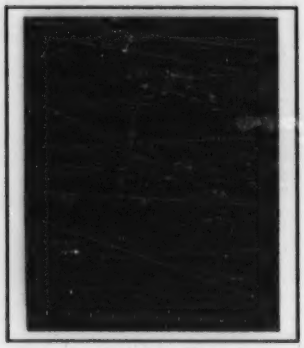


Fig. 6. Cross-section of globular embryo. Fig. 7. Globular embryo. Fig. 8. Heart-shaped stage embryo.



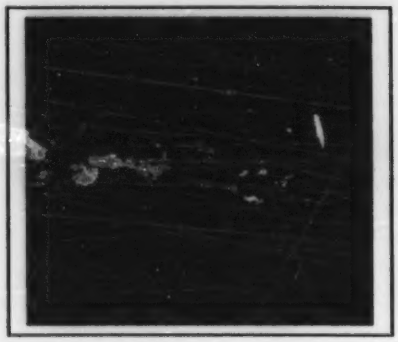


Fig. 9 and 10. Torpedo-shaped stage embryos.

d) Composition of the culture medium

The composition of the medium plays a major role in androgenesis for microspore initiation as well as the development of embryos. Important components are carbon sources and concentration, nitrogen sources and concentration, vitamins, growth regulators, pH and the presence or absence of gelling agents. Based on a survey of the literature and a large number of preliminary experiments, two initiation media were developed, CIM-1 and CIM-4 (Table 2).

Results showed that both media were effective for inducing globular embryos. Depending on genotype and other conditions, up to 76 globular embryos per square centimeter were obtained indicating an excellent potential for androgenesis of chickpea. Efforts then focussed on conversion of the globular to heart-shaped and then torpedo-shaped stage embryos by using different combinations and concentrations of growth regulators (BAP, zeatin, GA_3, ABA), growth regulator free medium, and anti-auxins. Furthermore, embryos were also exposed to medium with different osmotic potential, charcoal, polyamines, silver

Table 2. Composition of two chickpea induction media.

Medium	CIM-1	CIM-4
MS[1] macro	Yes	Yes
MS[1] micro	Yes	Yes
B5[2] vitamins	Yes	Yes
Sucrose	30 g/L	30 g/L
2,4-D	1.00 mg/L	1.00 mg/L
Picloram	0.25 mg/L	0.25 mg/L
BAP	0.10 mg/L	-
TDZ	-	2.20 mg/L
PH	5.8	5.8
Phytagel	3.0 g/L	3.0 g/L

[1] MS = Murashige and Skoog 1962. Phys. Planta 15: 473-497
[2] B5 = Gamborg *et al.* 1968. Exp. Cell Res. 50: 151-158.

nitrate, silver thiosulfate, acetyl salicylic acid and cold treatment. In addition, the effect of different culture vessels on embryogenesis was also examined. However, regardless of the treatment, globular embryos callused in all experiments and embryos developed only rarely to the heart-shaped or torpedo-shaped stage indicating that the carry-over effect of 2,4-D could not be eliminated.

Subsequent experiments then involved shorter exposure times (less than 7 days) as well as lower concentrations of 2,4-D. However, these experiments resulted in either considerably fewer embryos or no embryo development at all.

Hence, experiments were conducted using other growth regulators (1, 2, 3 mg/L IAA; 1, 2, 3, 4 mg/L NAA; 0.5, 0.75, 1.0, 2.0 mg/L Picloram; 1, 2, 3, 4 mg/L 2,4,5-T) instead of 2,4-D. These experiments are currently in progress. Preliminary results indicate that IAA might be a possible alternative for 2,4-D. IAA is quickly broken down within the embryo by exposure to light or room temperature.

e) Culture environment

Isolated microspores were incubated at 25 or 30°C in the dark. The higher temperature did not improve induction but tended to result in problems with mold contamination. The effect of light or absence of light during the initial incubation period was also tested. Exposure to light during the first 7 days of culture usually resulted in a decrease in the number of globular embryos. Currently, cultures are incubated at 25°C in the dark for about 10 days or until globular embryo development can be observed. The plates are then transferred to an incubator with a 16-hour photoperiod, a low light intensity, and a temperature of 23°C.

Preliminary experiments investigated the effect of gas composition on embryogenesis by manipulating the ethylene concentration via culture vessels as well as through the addition of polyamines, silver nitrate, and silver thiosulfate to the medium. None of these treatments had any positive effect on embryo initiation or development.

Determination of the optimal microspore density was also addressed. At this point, a minimum density of 100,000 microspores per ml seems to be required since any lower densities resulted in a lack of embryogenesis. However, an upper limit or an optimum density has not yet been determined.

4.4 Milestone: Evaluating factors influencing embryo culture response

a) Media constituents

Once embryos have been induced, they often require different media and growing conditions for further development. As outlined in subsection 2 d), a large number of embryo development media and conditions were tested. However, all embryos callused and rarely were heart-shaped or torpedo-shaped embryos obtained. It seemed that the residual effect of the 2,4-D in the initiation medium could not be removed from the embryos. However, it can only be concluded that the embryo development media tested were not effective given the parameters under which they were tested. It does not imply that they might not be effective if embryos can be obtained with growth regulators other than 2,4-D.

Hence, at this point the research has re-focused on the initiation medium and the constituents of the embryo development media will be re-assessed once a better initiation medium has been developed.

b) Physical factors

Chilling of globular embryos and exposure to high osmotica were tested but had no effect probably due to the 2,4-D problem.

c) Desiccation

Desiccation of embryos has not been attempted due to the lack of embryos in the appropriate stages.

4.5 Milestone: Regeneration

a) Plantlet regeneration from embryos or calli

At this point, embryos cannot be converted into plantlets. Hence, this part of the project could not be completed.

b) Chromosome doubling of microspores, embryos, calli, or plantlets

In a preliminary test microspores were exposed for 24 or 48 hours of colchicine (25 mg/L) resulting in a negative impact on embryogenesis. This part of the project was discontinued when DAPI staining of microspores showed that androgenesis in chickpea also involved symmetrical microspore nuclei divisions (see section 2c). In barley, these symmetrical nuclei divisions resulted in a high rate of spontaneous chromosome doubling and colchicine treatment was usually not required.

4.6 Conclusions

1. Androgenesis is also possible in chickpeas.
2. The most responsive microspore stage is the mono-nucleate stage.
3. There are genotypic differences between cultivars as well as interactions between genotype and environmental factors.
4. The first indicator of androgenesis is the symmetrical microspore nuclei division similar to other crops.
5. The minimum density was found to be 100,000 microspores per ml.
6. Embryos developed directly from microspores without an intervening callus phase.
7. Embryo development followed the usual stages from pro-embryo to globular and occasionally, heart-shaped and torpedo-shaped embryos.
8. The highest number of embryos obtained under sub-optimal conditions was 76 embryos per square centimeter indicating that androgenesis has excellent potential in chickpea.
9. The growth regulator 2,4-D is very effective for induction of embryos, however, its effect cannot be removed resulting in embryos that continue to callus.
10. At this point, IAA seems to be an alternative for 2,4-D. Material obtained from the initial experiment has not shown any signs of callusing to date.

4.7 Future Directions

Androgenesis is a complex process that requires all steps to function at least to some extent in order to obtain embryo and plant development. To develop such a system is extremely laborious and time consuming because interactions between the different steps requires re-checking of all components if a change has been made to the protocol.

Current research involves re-testing basal medium composition, optimizing the microspore density at the time of plating as well as varying the IAA concentration in the induction medium. However, avoidance of the use of growth regulators in the induction phase would be a preferable solution. This could be accomplished by using stress pre-treatments. Hence, experiments are underway to re-examine cold or heat stress, carbon starvation, carbon source and osmolarity of the medium, alone or in combination with each other.

4.8 Outputs and Deliverables - Posters

Lulsdorf, M; T. Dament; J. Croser; T. Warkentin; A. Vandenberg 2001. Development of microspore culture technology for the production of doubled-haploid lines of chickpea (*Cicer arietinum* L). NAPIA meeting, Fargo, North Dakota, USA.

Lulsdorf, M; A. Ferrie; J. Croser; T. Warkentin; A. Vandenberg 2002. Doubled-haploid technology for pulses. Pulse Days 2002, Saskatoon, Sask., Canada.

5 Personnel

A personnel listing will be supplied as part of a supplementary report accompanying the expense statement to be submitted by the University of Saskatchewan.

6 Equipmment

No equipment was purchased.

7 Innovation Fund Acknowledgment

The Agri-Food Innovation Fund was duly acknowledged verbally or through use of the AFIF logo at numerous oral presentations and in all publications and posters developed through the project funds.

8 Project Public Information Materials

A CD with copies of posters will be supplied.

9 Expense Statement

This will be supplied by the University of Saskatchewan Business Office.

Development of Microspore Culture Technology for the Production of Doubled-Haploid Lines of Chickpea (*Cicer arietinum* L.)

Monika Lülsdorf, Tim Dament, Janine Croser, Tom Warkentin, and Bert Vandenberg
Crop Development Centre, University of Saskatchewan,
Saskatoon, SK, S7N 5A8 Canada

ABSTRACT

Isolated microspore culture is a technique employed in a number of important field crops for the induction of instant homozygosity in breeding lines. This project investigated the application of this technology to chickpea (*Cicer arietinum* L.). At this point in our research, embryos can be induced directly from microspores without an intervening callus phase. Embryo development followed the usual stages from pro-embryo through globular and 'heart shaped' stages and sometimes to the cotyledonary stage. Our research is now focussed on obtaining mature embryos and their conversion into plantlets.

Introduction

In the past 4 years, chickpea production in western Canada has grown from 27,000 acres to an estimated 1,300,000 acres in 2001 (Cleary 2001) with over 90% being produced in Saskatchewan (Patterson 2001). Hence, improving chickpea cultivars is a major objective of the CDC Pulse Breeding Program. One approach is the doubled-haploid technology which is now commonly used in crops such as canola (e.g. Ferrie *et al.* 1995) and barley (e.g. Devine and Marten 1998). Plantlets developed from microspores are haploid and, upon doubling, a completely homozygous fertile plant can be produced in one generation. Phenotypic selection for quantitatively and particularly qualitatively inherited traits is much easier having doubled-haploid populations. In addition, molecular-marker assisted selection also becomes more efficient.

Materials and Methods

Cultivars Bumper, Garnet, Chico, Yuma, Myles, Desiray and several other CDC breeding lines were screened using a Murashige and Shoog (1962) based medium. Applications of stress pre-treatments such as heat, cold, and/or mannitol were also conducted in order to improve induction.

Fig. 1. Uni-nucleate chickpea microspore prior to mitosis using DAPI staining.

Fig. 2. Microspore after mitosis showing 2 similar nuclei using DAPI staining.

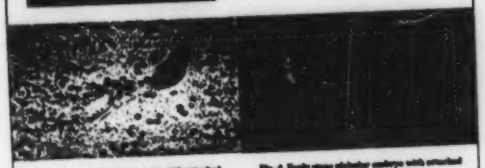


Fig. 3. Very early stage pro-embryo with attached sporoplast (acetocarmine staining).

Fig. 4. Early stage globular embryo with attached sporoplast (acetocarmine staining).

Results and Discussion

1. Induction of Embryogenesis

Embryos could be induced from most cultivars or breeding lines using isolated microspore culture. Stress pre-treatments did not improve induction. Similar to barley or canola, uni-nucleate microspores (Fig. 1) underwent mitosis and produced two similar nuclei (Fig. 2). These nuclei continued to divide and developed into pro-embryos with sporoplasts within a few days of culture (Fig. 3).

2. Development of Embryos

Embryo development during endrogenesis followed the usual stages from pro-embryo (Fig. 3) through globular (Figs. 4 - 7) to heart-shaped stage (Figs. 8 and 9). Cotyledonary stage embryos (Fig. 10) were only rarely obtained.

Fig. 5. Cross-section of globular stage chickpea embryos using acetocarmine staining.

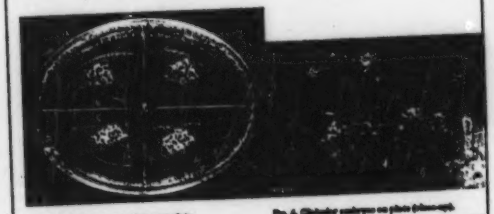


Fig. 7. Globular stage embryos on plate.

Fig. 6. Globular embryos on plate (close-up).

3. Conversion of Embryos

Conversion of cotyledonary embryos into plantlets has not yet been accomplished. Testing embryo development media using various hormones, osmotics and/or ethylene inhibitors are in progress.

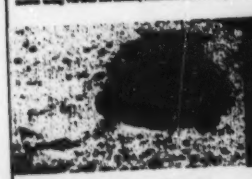


Fig. 8. Early "heart-shaped" stage embryo using acetocarmine staining.

Fig. 9. Heart-shaped stage embryo with sporoplast using acetocarmine staining.

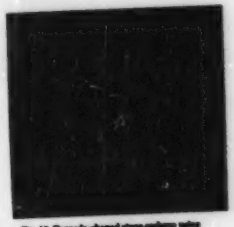


Fig. 10. Torpedo-shaped stage embryo using acetocarmine staining.

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We thank Kara Allen and Gordon Rudoczyjeck for their technical assistance.

Exploitation of Wild Species of Lentil for Better Disease Resistance in Canadian Lentil Varieties

[1]Tullu, A., L. Buchwaldt[2], T. Warkentin[1], M. Lulsdorf[1], S. Banniza[1], B. Taran[1], F. Ahmad, A. Sarkar, Al Slinkard[1], B. Barlow[1], C. Breitkrutz[1], S. McHale and B. Vandenberg[1]

[1]Crop Development Centre, University of Saskatchewan, Saskatoon, SK, S7N 5A8 Canada; [2]AAFC, Saskatoon, SK, S7N 0X2 Canada; [3]Botany Dept., Brandon University, Brandon, MB; [4]ICARDA, Tel Hadya, Syria

Introduction

- Lentil plays an important role in crop rotation and farm economics of the Canadian prairies. Its low fertilizer requirement and ability to fix nitrogen for the following crop makes it environmentally friendly and an important crop in sustainable agriculture.

- The crop is attacked by two major diseases (anthracnose and ascochyta) that can cause 100% loss in the worst scenarios. The Crop Development Centre (CDC) breeding program has up to now only used germplasm resources available in the cultivated lentil to develop new varieties with resistance to these diseases. However, the available cultivated germplasm lacks sufficient genetic variation for resistance to anthracnose and ascochyta diseases.

- In lentil, attempts to exploit wild species are a recent endeavour. Some work on ascochyta is done at International Center for Agricultural Research in the Dry Areas (ICARDA). Since anthracnose is only a major lentil disease in North America, no work has been done to improve resistance to this disease elsewhere.

- Wild species of many crops are known to carry many disease resistance genes lacking in the cultivated crop. We began the search for anthracnose and ascochyta resistance in 480 accessions of the six wild species of lentil (world collections), of which two can be easily crossed with the cultivated type.

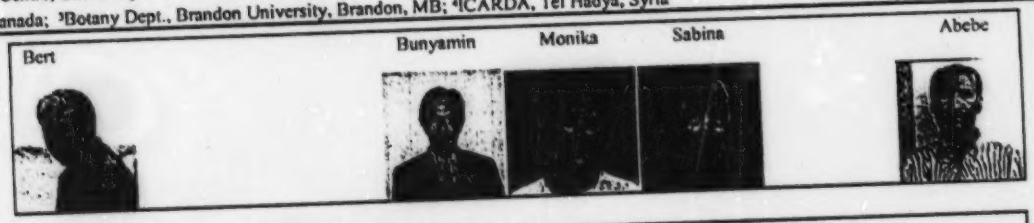


Figure 1. Field Experiment: (A). screening wild germplasm for resistance to anthracnose, (B) Resistance in L. ervoides, (C) susceptibility in L. orientalis, and (D) resistance in L. nigricans accessions.

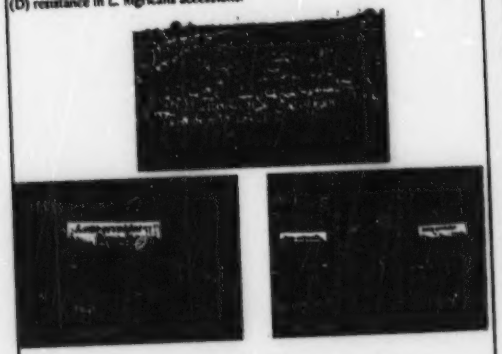
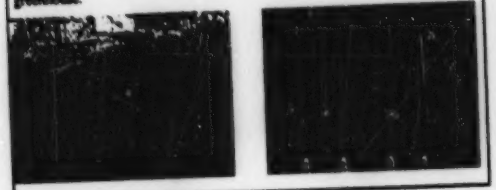


Figure 2. Indoor screening of wild germplasm: (D) L. orientalis for resistance to anthracnose, and (E) resistant wild germplasm identified in the greenhouse.

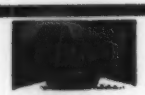


Summary and Coclusion

- Preliminary results indicate that there is a field resistance in many L. ervoides accessions; some L. nigricans and very few L. orientalis accessions to anthracnose disease.
- There is a higher level of resistance in many of the *Lens ervoides* accessions. However, this species is not crossable with the cultivated lentil. We need to use embryo rescue for successful transfer of genes to the cultivated species.
- If successful, the resulting germplasm developed from the interspecific crosses will be used by lentil breeders at the CDC to develop better varieties with resistance to anthracnose and ascochyta. Lentil growers, exporters and customers will benefit from improved varieties.

Acknowledgement

- Help from pulse crops technicians and crew members is highly appreciated.
- We acknowlege Saskatchewan Pulse Growers for funding this project.



Monika Lülsdorf

Bert Vandenberg

Tom Warkentin

Doubled-Haploid Technology for Pulses

Monika Lülsdorf[1], Alison Ferrie[2], Janine Croser[3], Tom Warkentin[1], and Bert Vandenberg[1]

[1]Crop Development Centre, University of Saskatchewan, Saskatoon, SK, S7N 5A8 Canada

[2]Plant Biotechnology Institute, NRCC, Saskatoon, SK, Canada

[3]CLIMA, University of Western Australia, Nedlands, WA, Australia

National Research Council Canada

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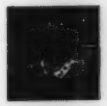


Alison Ferrie

ABSTRACT

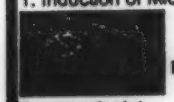
Doubled-haploid technology using anther or microspore culture is a powerful breeding tool for the improvement of crops. Microspores are immature pollen grains that contain only a haploid set of chromosomes. When they are cultured under sterile conditions, they can develop into embryos and subsequently be regenerated into haploid plants. Spontaneous or induced doubling of chromosomes will then allow regeneration into completely homozygous diploid plants. The main advantage of this technology is that one can rapidly fix traits in the homozygous condition, eliminating 3 to 4 generations of selfing. In addition, selection becomes more efficient, recessive genes can be more easily identified, and genetic manipulation is also easier at the haploid level.

This technology is now commonly used in crops such as canola, barley and wheat for the development of advanced breeding lines and cultivars (e.g. Quantum, Q1, Duplo, Mingo, or McKenzie). However, this technology is not yet available for pulses. Research is in progress at the Crop Development Centre (University of Saskatchewan) and at the Plant Biotechnology Institute (NRCC Saskatoon) to develop this doubled-haploid technology for pea, chickpea, and lentil. Microspore-derived embryos have been obtained from pea and chickpea. However, more research is required in order to develop an efficient and reliable system for these crops.

The availability of doubled-haploid technology for pulses would have a significant economic impact. Speeding up the breeding process for pulses and improving selection would allow pulse growers faster access to improved cultivars.

Pea

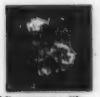
1. Induction of Microspores



Microspore nucleus just prior to first division.

Microspore with 2 symmetrical nuclei

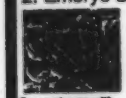
Nuclei continue to divide.



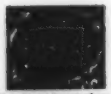
Microspore with multiple nuclei.

Microspores are immature pollen grains that normally follow the gametophytic pathway and develop into mature pollen. However, under stress conditions such as heat or cold, they can be induced to follow a different (sporophytic) pathway that leads to the development of embryos. Under stress, microspore nuclei undergo several symmetrical divisions (see Figs. 1, 2, 3, 4 for pea, Figs. 11, 12 for chickpea) with associated cell divisions.

2. Embryo Development



Pro-embryos with suspensor in early globular stage.

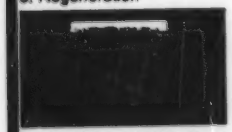


Globular to heart-shape stage embryo.

Mature pea embryo on plate.

Growth regulators in the medium then induce those cells to differentiate and to develop into pro-embryo like structures (Fig. 5 for pea, Fig. 13 for chickpea). Further embryo development follows the same stages as in 'regular' embryogenesis from globular (Fig. 6 for pea, Figs. 14, 15, 16 for chickpea) to heart-shaped stage (Fig. 17 for chickpea), and to the cotyledonary stage (Fig. 7 for pea, Fig. 10 for lentil, Fig. 18 for chickpea).

3. Regeneration

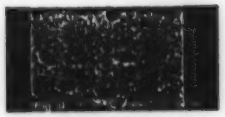


Pea plantlet regenerated from microspore.

Mature embryos can then be regenerated into plantlets (Fig. 8 for pea).

Lentil

1. Induction of Microspores



Isolated lentil microspores

2. Embryo Development

Potential embryo developed from isolated lentil microspore

Acknowledgement

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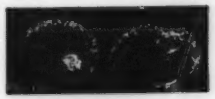
We thank Helen Baker, Kara Allen, Tim Dennis, and Gordon Rudzewicz for their technical assistance.

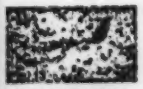
Chickpea

1. Induction of Microspores



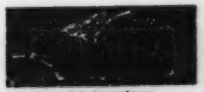
Mono- and bi-nucleate microspore. Note symmetrical division i.e. 2 similar nuclei.

2. Embryo Development



Early stage pro-embryo with attached suspensor.

Early globular embryo with attached suspensor.



Late stage globular embryos.

Globular embryos on plate.

Heart-shaped stage embryo.

Torpedo-shaped stage embryo.

Doubled-haploid technology for pulses is still in its early stages of development. Currently, we are able to obtain a large number of globular embryos from pea and chickpea within 7 days of culture. However, the switch from the globular stage to the mature embryo is still difficult to initiate and we rarely obtain plantlets. More research is required to improve the development of a microspore culture protocol for pulses. Results obtained from our experiments so far indicate that there is a potential for obtaining a protocol that will be efficient and cost effective.

Molecular Marker Techniques Applied to Pulse Crop Breeding: Assisted Selection for Disease Resistance

B. Tar'an, A. Tullu, C. Breitkreutz, A. Vandenberg, T. Warkentin & S. Banniza
Crop Development Centre, University of Saskatchewan, Saskatoon, Canada S7N 5A8

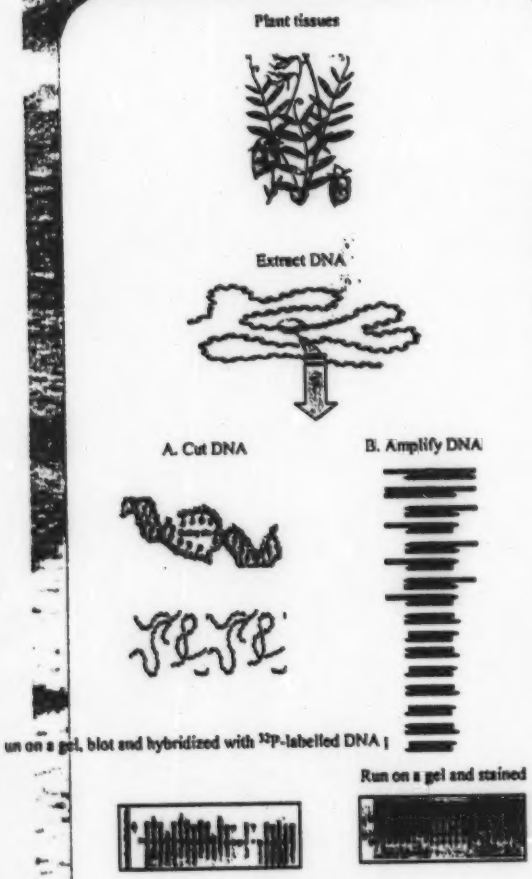


Fig. 1. DNA fingerprints are produced by cutting (A) or amplifying (B) DNA samples from biological organisms to give small DNA fragments that can be separated, according to their size, by moving them with an electric current through a porous slab-shaped gel. The DNA fragment patterns that result from this electrophoresis procedure resemble product bar codes.

Introduction

In plant breeding programs, breeders usually selected plants based on their visible or measurable traits known as phenotype, such as disease resistance, seed yield and earliness. However, these traits can be skewed depending on the influence of the field season and growing conditions. A large number of progeny are usually evaluated in replicated trials. In addition, for some traits such as disease resistance, certain stages of plant growth are required for accurate screening. With the advent of molecular marker technology, a new breeding tool is available to make more accurate and effective selections in breeding programs.

Molecular Markers and Marker-assisted selection

Molecular markers are pieces of genetic code (DNA; Fig. 1) that are associated with traits important in crop varieties. Because they are associated with the important traits (Fig. 2), they can be used to assist selection (marker-assisted selection = MAS) in breeding programs. The presence or absence of genetic markers is stable regardless of the environment that the breeding lines are grown in. Thus, a strong association of a marker with a trait will allow the breeder to more effectively select for the trait regardless of the environmental conditions. Also, new methods of DNA analysis are quick, and because they require very little plant material, they can be done during the early stages of plant growth. The advantages of MAS for disease resistance in particular are following:

- Faster and cheaper
- Reproducible
- Absence of pathogen
- Expressed earlier
- Enable gene pyramiding

Application of MAS in pulse crop breeding program at the Crop Development Centre

DNA marker technology is being increasingly used as a tool for crop improvement at the CDC pulse breeding program. This technology will undoubtedly provide improved efficiency in screening for agronomic traits. Over the past two years, we have been working to identify molecular markers for genes that make lentil and chickpea plants resistant to ascochyta blight and anthracnose (Fig. 3). In addition, MAS for resistance to common bacterial blight (CBB; Fig. 4) and bean common mosaic virus (BCMV) of beans, and lodging resistance in pea has been initiated at the breeding program.

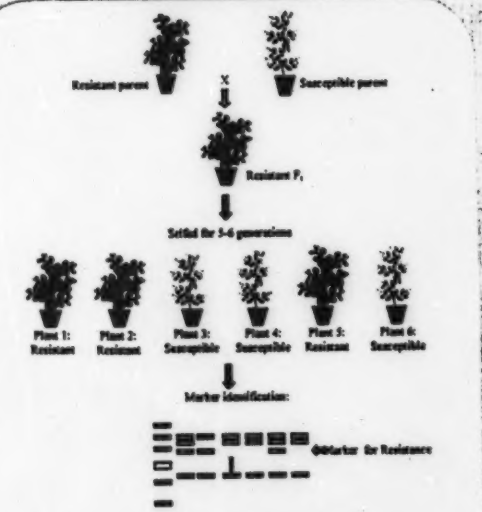


Fig. 2. The process of marker identification for disease resistance in pulse crops. Associations between individual DNA fragments and genes controlling disease resistance can be determined by studying the segregation patterns of the DNA fragments, and the genes of interest in plant breeding populations. DNA fragments frequently found in plants that express the trait and are absent from plants that do not have the trait, are called DNA markers.

Fig. 3. Segregation of molecular markers for resistance to ascochyta blight in chickpea (A; AF_{1000}) and lentil (B; $OPB18_{1000}$). The disease scores (on a scale of 0 to 9) for each line are indicated.

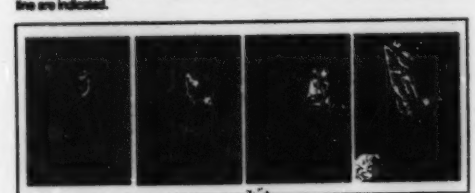


Fig. 4. Interaction of two loci, tagged by R7313 and R4885 markers, respectively, for resistance to CI